EFFECT OF OVARIECTOMY AND ADMINISTRATION OF OVARIAN HORMONES AND TESTOSTERONE ON NICOTINIC ACID METABOLISM OF RATS[1,2]

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In a previous report on the metabolism of nicotinic acid in pregnancy (Lojkin, Wertz and Dietz, '52), the theory was offered that the increased urinary excretions of metabolites of nicotinic acid observed in pregnant women and rats might possibly be attributed to the changes that occur in endocrine functions during pregnancy. Although the secretory activity of several hormones changes during pregnancy, it appeared of special interest to investigate the role of estrogen and progesterone, which are present in greatly increased concentrations during pregnancy, and of testosterone, which under certain conditions can inhibit (Turner, '55) or be inhibited (Martin et al., '55; Pincus and Dorfman, '55) by ovarian hormones.

A few short preliminary experiments were designed to observe how these hormones affect the urinary excretion of N′-methylnicotinamide (MNA) and the acid-hydrolyzable metabolites of nicotinic acid (NA) by the female rat. These tests indicated that the administration of testosterone propionate produced an immediate response, which consisted of

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an initial decrease in the output of MNA and NA, followed by a subsequent rise to levels exceeding the initial values (Dick, '52). Such changes had been reported previously for dogs by Beher and Gaebler ('50) and later for rats (Beher et al., '52). The preliminary experiments with progesterone, estrone, and a combination of these two hormones, on the other hand, gave inconclusive results. There were no definite changes in the excretion of MNA and NA immediately after the injections, but there were indications of a delayed increase in the output of both these metabolites (Dick, '52; Lojkin and Dick, '52). These results indicated that the experimental period should be lengthened, and suggested the possible interference of the varying amounts of ovarian hormones which are normally present in an animal.

To eliminate any interference from the ovarian hormones produced by intact animals, ovariectomy was performed on a group of rats which were subsequently used to extend the preliminary study. It was found that a combination of 500 μg of progesterone and 2 μg of estrone was more effective in elevating the MNA excretion levels of rats than either hormone given singly at the same dosage. Furthermore, the percentage increase in the MNA output, under the conditions of these experiments, was approximately twice as high in the ovariectomized rats as in the intact rats (Lojkin and Wertz, '54). The effect of ovariectomy on the excretion of the metabolites of nicotinic acid, and a comparison of the responses of both ovariectomized rats and intact rats to the administration of ovarian hormones and testosterone propionate apparently justified further investigation. In view of the fact that tryptophan, which may be converted to nicotinic acid, is excreted in increased amounts during pregnancy (Miller et al., '54; Sheft and Oldham, '52; Wallraff et al., '50) it was also of interest to observe the effect of ovarian hormones on the excretion of tryptophan.

This report presents the results of an investigation designed to observe and compare the effects produced on the niacin metabolism of albino rats by changes in hormonal activity,

induced by ovariectomy and by injections of a combination of progesterone and estrone, and testosterone propionate.

METHODS AND PROCEDURES

Adult female albino rats, weighing approximately 300 gm, from a strain originally obtained from the Connecticut Agricultural Experiment Station, were used in this investigation. They were fed ad libitum a stock breeder's ration consisting of 10% meat scraps, 60% whole wheat, and 30% whole milk powder, to which 1.5% NaCl was added. This ration contained 44 μg of nicotinic acid and 1.5 mg of tryptophan per gram. The animals were maintained in metabolism cages continuously throughout the experimental periods. Records of the daily food intake were kept, and the weights of the rats were recorded at intervals during the study. Twenty-four-hour urine samples were collected under toluene, diluted to volume, filtered, and stored frozen at — 20°C. until analyzed. Determinations of MNA were made on each individual daily sample of urine by the fluorometric method of Huff and Perlzweig ('47). Representative samples were analyzed for nicotinic acid by the microbiological method of the Association of Vitamin Chemists ('51). Samples from the ovariectomized rats in experiment I were analyzed for tryptophan microbiologically with *Leuconostoc mesenteroides* as the test organism by the method of Steele et al. ('49).

Experiment 1. Six adult female rats were placed in individual metabolism cages and 24-hour urine collections made for from 5 to 12 days. At the end of this period, the rats were ovariectomized. In 4 to 6 weeks after the operation, the 6 ovariectomized rats as well as 6 intact rats were placed in individual metabolism cages and 24-hour urine collections made for a pre-injection period of at least 9 days, an injection period in which a combination of 4 mg of progesterone and 0.5 μg of estrone in sesame oil was administered intramuscularly for 10 consecutive days, and a post-injection period of sufficient duration for the excretion of MNA to approach the pre-injection level. This dosage of progesterone and estrone

was selected for use in this experiment because it had been reported by Nelson and Evans ('54) to be effective in maintaining pregnancy in rats on protein-free diets. The post-injection period varied from 15 to 35 days for the individual rats. To determine whether the response to a second series of injections would be the same as the response to the first injections, this experiment was repeated on two of the ovariectomized rats several weeks after the first hormonal administrations were discontinued.

Experiment 2. Three ovariectomized and two intact rats were placed in individual metabolism cages and 24-hour urine collections made for a pre-injection period of at least 12 days, for an injection period in which 8 mg of testosterone propionate in sesame oil were administered intramuscularly for 5 consecutive days, and for a post-injection period of 12 days.

RESULTS

Effect of ovariectomy and administration of progesterone and estrone

The values for the urinary excretion of MNA and NA and for the food intake of the intact and ovariectomized rats, treated with a combination of 4 mg of progesterone and 0.5 μg of estrone, are summarized in table 1. The data represent the average of the results obtained from analyses of individual 24-hour urine samples for the pre-ovariectomy period, the pre-injection period, and for 5-day periods during and after the administration of the hormones. The average daily excretion of MNA, expressed as percentages of the initial values are presented in figure 1.

Marked individual variations were observed in the excretion of the metabolites of nicotinic acid by the different rats, with average values ranging for MNA from 165 to 342 μg/24-hour and for NA from 83 to 167 μg/24-hour for 12 intact, untreated animals. Variations in the excretion of MNA and NA, not only for rats of different strains (Guild et al., '54), but

also for individual rats of the same strain, have been previously reported (Perlzweig et al., '43; Lojkin et al., '52).

There were also quantitative variations in the responses of individual animals to both ovariectomy and hormonal therapy. However, the trend in the changes of the excretion of the metabolites of nicotinic acid induced by these treatments was similar in all the animals studied. Ovariectomy

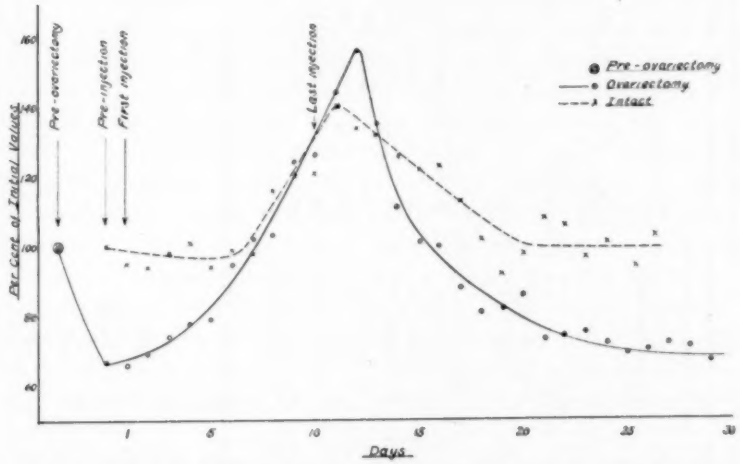

Fig. 1 Average daily urinary excretions of N'-methylnicotinamide for intact rats and ovariectomized rats injected with a combination of 4 mg of progesterone and 0.5 μg of estrone for 10 consecutive days, expressed as percentages of initial values. (The average initial value for the intact rats is 196 μg/24-hour; the average initial pre-ovariectomy value is 226 μg/24-hour.)

resulted in a marked drop in the MNA and NA output of all the rats. The decreases averaged 33 and 23% of the initial values for MNA and NA, respectively, and were statistically significant at the 1% probability level. The excretion of the metabolites of nicotinic acid by the ovariectomized rats remained unaltered during the first two days of hormonal administration. A gradual augmentation was noticeable in most of the rats on the third day (fig. 1). The MNA and NA excretion approached the initial pre-operative levels on the 7th

TABLE 1

Average urinary excretion of N'-methylnicotinamide (MNA) and the acid-hydrolyzable metabolites of nicotinic acid (NA) by ovariectomized and intact rats injected with a combination of 4 mg progesterone and 0.5 μg estrone for 10 consecutive days

		OVARIECTOMIZED RATS			INTACT RATS		
NO. OF RATS Period	Days	6 MNA	5 NA	6 Food intake	6 MNA	4 NA	6 Food intake
		μg/24 hr.		*gm/day*	*μg/24 hr.*		*gm/day*
		Initial values					
Pre-ovariectomy		226 ± 24 [1]	134 ± 14	13			
		Post-ovariectomy values					
Pre-injection		152 ± 16	103 ± 13	15	196 ± 11	103 ± 11	13
Injection	1–5	170 ± 16	106 ± 13	13	186 ± 14	101 ± 10	12
	6–10	253 ± 24	152 ± 20	14	219 ± 23	128 ± 15	14
Post-injection	11–15	293 ± 25	159 ± 22	13	259 ± 20	120 ± 11	14
Maximum values		355 ± 32	199 ± 29	14	315 ± 19	150 ± 13	13
	16–20	193 ± 15	105 ± 16	14	204 ± 11	92 ± 9	11
	21–25	162 ± 17	95 ± 15	15	204 ± 11	96 ± 12	12
	26–30	155 ± 16	94 ± 15	14			
		Probability levels of significances of differences among periods					
		%	%		%	%	
Initial non-operated							
vs. ovariectomized		$p \leq 1$	$p \leq 1$				
vs.	11–15	$p \leq 2$			$p \leq 1$		
vs. maximum		$p \leq 1$	$p \leq 5$		$p \leq 1$	$p \leq 1$	
Ovariectomized pre-injection							
vs.	11–15	$p \leq 2$					
vs. maximum		$p \leq 1$	$p \leq 1$				

[1] Standard error of the mean.

and 5th day, respectively, and reached maximum values for MNA on the 11th or 12th day and for NA on the 9th to 12th day after the first injection. Declines in the excretion values started after the 11th to 15th day and continued for periods which varied in duration for the individual animals. On the average, the values returned to levels approaching the pre-injection values by the 25th day.

Administration of 4 mg of progesterone and 0.5 μg of estrone to intact rats (fig. 1) induced changes in MNA and NA excretion which followed the general trend observed in ovariectomized rats, although a longer period of time was required for the occurrence of the response. On the average, the MNA and NA values started to rise after the 8th and 6th day, respectively, of hormonal administration. Although the results from the individual rats tended to fluctuate rather than to increase steadily from day to day, the mean values changed steadily from one period to another (table 1). The daily excretion values reached their maximum levels for the different rats after intervals that varied in duration from 11 to 15 days for MNA and from 8 to 12 days for NA. The excretions approached the pre-injection levels during the 20-to-25-day period.

Results of a comparison of the excretion values during the different periods of the experiment (table 1) indicated that the maximum MNA excretion of the ovariectomized injected rats exceeded the pre-injection values by 203 μg or 134% and the pre-ovariectomy values by 129 μg or 57%. The average for the 5-day period (11th to 15th day), in which the maximum response occurred, exceeded the pre-injection levels by 141 μg or 93% and the pre-ovariectomy levels by 67 μg or 30%. In the intact rats the maximum MNA values and the average values for the 5-day period during which the maximum MNA excretion occurred exceeded the average intial pre-injection values by 119 μg or 61% and 63 μg or 32%, respectively. All these differences were statistically significant.

A highly significant correlation between the MNA and NA excretion was found in each of the rats. In both the ovari-

ectomized and the intact rats the changes in NA excretion induced by the administration of ovarian hormones followed the same trend as the corresponding changes in MNA excretion, but preceded them by one or two days. The percentage of the decrease in NA excretion resulting from ovariectomy and the percentage of the increase induced by hormonal administration were smaller than the corresponding changes in MNA excretion. The maximum NA excretion levels of the ovariectomized rats exceeded their average pre-injection values by 96 μg or 93% and their initial pre-operative values by 65 μg or 48%, and the intact animals' maximum NA excretion exceeded their average initial values by 47 μg or 46%.

A repeated series of injections with the same dosage of progesterone and estrone, administered to two ovariectomized rats several weeks after the previous injections had been discontinued, resulted in a similar pattern of response in MNA excretion. However, the initial pre-injection values for the excretions by the two ovariectomized rats, the order of magnitude of the increases, as well as the percentage increase over the initial values were smaller than in the first series of injections on the average by 30 μg, 80 μg and 40%, respectively. This finding suggests the possibility that under the conditions of these experiments ovarian hormonal administration might have reduced the animals' ability to respond to further hormonal treatment.

Results of the analysis for the tryptophan in uncombined form in the urine of the ovariectomized rats indicated an average excretion of 50 μg per 24 hours for the pre-injection period. There were no consistent or significant changes throughout the experimental period.

The average weight of the rats to be ovariectomized was 311 gm before ovariectomy was performed. It increased 15% during the period after ovariectomy, averaging 357 gm at the beginning of the pre-injection period and remained at that level throughout the experiment. The average weight of the intact rats was 309 gm at the beginning of the pre-injection period and remained unchanged. Average daily food intakes

of the two groups of rats varied from 11 to 15 gm for the different periods, but indicated no consistent trend from period to period. Although the initial weights of the two groups of rats were the same, the average initial excretion values for MNA and NA were somewhat higher for the group of rats that were to be ovariectomized than for the group of normal intact rats. These experiments did not indicate any significant correlations between the metabolites of nicotinic acid excreted and the weights, or food intakes, of the rats.

TABLE 2

Average urinary excretion of N'-methylnicotinamide (MNA) and the acid-hydrolyzable metabolites of nicotinic acid (NA) by ovariectomized and intact rats injected with 8 mg testosterone propionate for 5 consecutive days, expressed as percentages of initial values

		OVARIECTOMIZED RATS			INTACT RATS		
NO. OF RATS		3	3	3	2	2	2
Period	Days	MNA	NA	Food intake	MNA	NA	Food intake
		μg/24 hr.		*gm/day*	*μg/24 hr.*		*gm/day*
		Initial values			Initial values		
Pre-injection		140	78	13	161	97	14
		% of initial values[1]			% of initial values[1]		
Pre-injection		100	100	100	100	100	100
Injection	1–5	57	74	104	50	78	109
Post-injection	6–8	83	104	110	49	89	100
	9–11	110	117	104	61	96	104
	12–14	124	131	110	75	103	106
	15–18	118	148	110	110	121	112

[1] Averages of the individual percentages.

Effect of testosterone

Data regarding urinary excretions of MNA and NA as well as the food intake of ovariectomized and intact rats injected with testosterone propionate are presented in table 2. The figures represent the averages of the results obtained from analyses of separate 24-hour samples for each rat for the pre-injection period, a 5-day injection period, and 3-day post-

injection periods, and are expressed as percentages of the initial values.

Treatment of ovariectomized and of intact rats with testosterone propionate produced results in general agreement with those previously observed in these laboratories (Dick, '52) and also reported by other investigators (Beher et al., '52) for the intact animals. Administration of 8 mg of the hormone for 5 consecutive days induced an immediate sharp drop in the excretion of both MNA and NA in the ovariectomized as well as in the intact animals. The decrease of MNA excretion during the 5-day injection period averaged 43 and 50%, respectively, for the ovariectomized and the intact animals. In the ovariectomized animals the excretion of MNA started to increase immediately after the injections were discontinued, reaching the pre-injection values during the 9– to 11–day period and exceeding them on the 12th or 13th day. The MNA excretion of the intact animals, on the other hand, remained at lowered levels for a longer time and reached the initial values on the 13th or 15th day.

The percent of decreases in the excretion of NA resulting from the hormonal treatment of ovariectomized rats was smaller than the corresponding changes in MNA levels. The correlation between the amounts of MNA and NA excreted by each rat was statistically significant. However, the average NA excretion values returned to their initial levels two or three days earlier than the corresponding MNA excretions.

The food intake of the animals changed little during the experiment. An average increase of 10 gm in the weights of the animals occurred during the injection period, and weights remained at the increased levels for the duration of the experiment. There was no correlation between the levels of excretion of nicotinic acid metabolites and food intakes, or weights of the animals.

DISCUSSION

Ovarian hormones appear to play a definite role in nicotinic acid metabolism of the female rat. Reduction of hormonal

activity induced by ovariectomy resulted in marked decreases in the excretion of both MNA and NA. The initial pre-ovariectomy MNA and NA excretion levels exceeded those of the ovariectomized rats by approximately 50 and 30%, respectively.

Increases in the amount of ovarian hormones present in the animal's body as the result of the administration of a combination of 4 mg of progesterone and 0.5 μg of estrone, induced definite increases in the output of nicotinic acid metabolites. In the rats whose MNA and NA excretion had been lowered by ovariectomy the total rise and the rate of augmentation of the excretion of these metabolites were especially pronounced.

The increase in the MNA and NA excretion of the ovariectomized animals became noticeable on the third day after the beginning of the hormonal administration. The MNA and NA values of these rats increased by approximately 50 and 30%, respectively, and thus reached the levels of their initial pre-operative excretion values, before the excretion of the intact rats began to rise. Subsequent elevations in the excretion values of the nicotinic acid metabolites proceeded at approximately equal rates in both groups of rats. The maximum MNA and NA excretion values reached by the ovariectomized rats exceeded their initial pre-ovariectomy levels of excretion by 57 and 48%, respectively, and those of the intact animals exceeded their initial MNA and NA values by 61 and 46%, respectively. These results indicate that under the conditions of these experiments, hormonal therapy could elevate the excretion of nicotinic acid metabolites to a certain definite per cent above the initial values, regardless of whether the hormones were administered to ovariectomized rats, which had low pre-injection excretion values, or to intact animals, which had higher pre-injection MNA and NA excretion levels.

In the study of the metabolism of nicotinic acid in pregnancy (Lojkin et al., '52), it was observed that urinary excretion of MNA and NA in pregnant rats increased gradually with the duration of pregnancy, and exceeded, during the

week of parturition, the pre-pregnancy levels by 90 and 56%, respectively. These increases, as might be expected, were higher than those resulting from the administration of progesterone and estrone because this hormonal treatment could not duplicate the complex changes in hormonal activity that occur in pregnancy. However, the fact that the concentration of progesterone and estrogen is greatly increased during pregnancy, and that a marked similarity is observed in the pattern of changes in the MNA and NA excretions, resulting from pregnancy or induced by hormones, strongly support the theory that the increased excretion of nicotinic acid metabolites in pregnancy can be at least partially attributed to changes in ovarian activity in the pregnant body.

The reactions responsible for the effect of ovarian hormones which lead to augmented excretion of nicotinic acid metabolites cannot be explained satisfactorily. However, some theories might be postulated on the basis of the data presented by Nelson and Evans ('54), who demonstrated that ovarian hormonal therapy, consisting of injections of progesterone and estrone, in doses equal to those used in these experiments, was effective in maintaining pregnancy in rats on protein-free diets. This finding, according to the authors, demonstrates a hormonal inadequacy in protein-deficient animals. It might also be interpreted as suggesting that the ovarian hormones affect the metabolism of protein, either directly or by stimulating or depressing the effect of other hormones or enzymes on protein metabolism. If the resulting changes in protein metabolism involved the breaking down of protein, it appears possible that tryptophan might be liberated for conversion to nicotinic acid. It is not known whether ovarian hormones can stimulate in any way the formation of coenzymes I and II or the release of nicotinic acid from the body reserves.

The ability of testosterone propionate to affect the urinary MNA excretion levels as well as the coenzymes I and II content of the livers and kidneys of rats has been reported by Beher et al. ('52). According to the findings in these labora-

tories, the decreases in the excretion of nicotinic acid metabolites resulting from injections of testosterone propionate, were of the same order of magnitude for the ovariectomized rats and for their intact controls. This similarity would indicate that the decreases could not be attributed, at least entirely, to inhibiting effects between the ovarian hormones and testosterone. However, the prolonged depression in the levels of MNA and NA excretion in the intact rats, compared to the very rapid return to higher levels of excretion in the ovariectomized rats, suggests that the presence of ovarian hormones may to a certain degree influence in some way, possibly indirectly, the effect of testosterone on the metabolism of nicotinic acid.

SUMMARY

Hormonal activity of female rats was changed by means of ovariectomy and by administration of ovarian hormones and of testosterone propionate.

In a group of rats that excreted on the average 225 μg/24-hour of N′-methylnicotinamide (MNA) and 133 μg/24-hour of the acid-hydrolyzable metabolites of nicotinic acid (NA), ovariectomy resulted in a mean decrease of 33 and 23%, respectively, in the urinary excretion of MNA and NA.

Ten daily injections of a combination of 4 mg of progesterone and 0.5 μg of estrone resulted in statistically significant rises in the excretion of the nicotinic acid metabolites. The maximum MNA and NA excretion by the ovariectomized rats exceeded their pre-injection excretion values, on the average, by 134 and 93%, respectively. The percentages of increases of the excretions of ovariectomized and intact animals over the levels of the initial excretions were of the same order of magnitude, regardless of whether the hormones were administered to the intact or the ovariectomized animals. The maximum rises averaged approximately 59 and 47%, respectively, for MNA and NA excretions.

There was a statistically significant correlation between the MNA and NA excreted by each of the experimental animals throughout the investigation.

The amounts of uncombined tryptophan excreted by ovariectomized rats apparently were not affected by the activity of the ovarian hormones.

Administration of testosterone propionate induced decreases in the excretion of nicotinic acid metabolites in all the injected rats. The difference between the percentage decrease in the excretions of the ovariectomized and the intact animals were not significant.

Evidence was provided in favor of the concept that the high concentration of ovarian hormones in pregnancy may be one of the factors responsible for the changes in the metabolism of nicotinic acid, which had been observed in the pregnant rat and in the pregnant women.

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THE INFLUENCE OF CHLORTETRACYCLINE AND VITAMIN B_{12}, ALONE AND IN COMBINATION, ON NITROGEN UTILIZATION BY GROWING SWINE[1]

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Numerous attempts have been made to define the mechanism by which antibiotics exert their growth-promoting effects in swine since it was discovered (Jukes et al., '50) that an antibiotic, aureomycin, was the cause of greatly increased gains of growing swine fed a crude "Animal Protein Factor" supplement. Control of disease level (Speer et al., '50), sparing of niacin (Powick et al., '50), sparing of methionine (Cunha et al., '49), and sparing of crude protein (Cunha et al., '50; Catron et al., '52; Burnside et al., '54) have all been proposed as explanations for the mode of action of antibiotics. Diets adequate in the B vitamins and containing 15 or 18% of crude protein were of equal value for weanling pigs in the presence or absence of supplemental oxytetracycline (terramycin), but a possible sparing effect of oxytetracycline upon protein requirement was not indicated (Hoefer et al., '52). It has been pointed out that for an antibiotic to exert a true sparing action on the protein requirement of pigs (Jensen et al., '55), it would be necessary for pigs fed low protein diets

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to show an increased nitrogen retention due to the antibiotic. The measure of response in most instances was rate of gain.

Two studies have been conducted using the nitrogen balance method (Brown et al., '52; Braude and Johnson, '53) and in neither case did chlortetracycline (aureomycin) cause a significant increase in nitrogen balance of growing pigs. It is possible that the protein content of the diets used in these investigations, 20.6 and 15.9% respectively, was so high as to mask a sparing action of chlortetracycline upon the protein requirement if it were present. In only one instance (Russo et al., '54) has significantly increased nitrogen retention of growing pigs been reported due to an antibiotic. A 12% crude protein diet was used by these investigators.

The present study was designed to determine the effect of chlortetracycline and vitamin B_{12}, alone and in combination, upon nitrogen metabolism of growing pigs when the experimental diets were fed at three levels of feed intake. Since one of the primary results of supplementing swine diets with antibiotics is an increase in feed consumption with a resultant increase of daily gains, three levels of feed intake were employed to obtain data to clarify the roles of chlortetracycline, vitamin B_{12} and level of feed intake in influencing nitrogen balance of growing swine.

MATERIALS AND METHODS

Growing barrow pigs which had been weaned at approximately 8 weeks of age were used in this study. Pigs which were assigned to two groups according to size and genetic background were randomly assigned to treatments. Random assignment was made of the barrows to feed intake level within treatment for each collection period. Individual pig weights were taken three or 4 days prior to placing pigs on collection and at the start and finish of each collection period. Feed intakes were based on an estimated average weight of the pig for a given collection period. Individual feedings were made twice daily. Nitrogen balance data were obtained, using preliminary periods of 10 or more days to accustom pigs to

diets and environment, adjustment periods of at least 8 days between collections from the same pig, 7-day periods for collection of feces and 5-, 6- or 7-day periods for collection of urine. All collections were made using adjustable cylindrical swine metabolism cages patterned after those designed by Bell ('48).

The basal diet consisted of the following by weight: ground yellow corn, 82.8; solvent soybean oil meal, 9.8; meat scraps, 4.9; steamed bone meal, 2.0 and iodized salt, 0.5. Adequate amounts of vitamins A and D, riboflavin, niacin, pantothenic acid and choline were included in the diet along with a trace element mixture. This diet contained 14.1% of crude protein based on nitrogen determinations of the basic ingredients. The basal diet was fed unsupplemented (treatment 1) and with the following levels of supplements indicated per kilogram of diet: 22 mg of chlortetracycline (treatment 2), 11 μg of vitamin B_{12} (treatment 3), or a combination of the above levels of chlortetracycline and vitamin B_{12} (treatment 4).

Each diet was fed at levels closely approximating 2.9, 3.5 and 4.1% of the live weight of the individual pigs during the different collection periods, thus making a total of 12 subtreatments. The daily feed intake of each pig, based on the predicted mean weight for the pig while on collection, was held constant during the last 4 days of the adjustment period and all of the collection period.

All data have been summarized to present animal weights as mean weight expressed in kilograms for the time that the individual pigs were on collection. These mean weights of the individual pigs have been reduced to $W_{kg}^{0.734}$ to eliminate insofar as possible the influence of difference in body weight, W, between different pigs and different periods (Brody and Procter, '32). This approach was recently used in studying nitrogen metabolism of pigs fed varying concentrations of dietary protein (Armstrong and Mitchell, '55). Armstrong and Mitchell fed their animals at a level of 5% of body weight raised to the power 0.90, while all pigs used in these investigations were fed on the basis of actual body weight.

Data are presented showing the average daily nitrogen metabolism of the pigs used and the mean daily nitrogen metabolism per unit of metabolic size ($W_{kg}^{0.734}$). All conclusions are based on daily nitrogen retention per unit of metabolic size. Data have been treated statistically using analysis of variance and covariance (Snedecor, '46).

RESULTS AND DISCUSSION

The average nitrogen mteabolism data for the pigs used on the various treatments and on the levels of feed intake within each treatment are shown in table 1. There was some tendency for pigs fed diets containing chlortetracycline to absorb more nitrogen at all levels of feed intake with the exception of the 2.9% level of feed intake on treatment 2. The pigs fed diets containing chlortetracycline, with or without added vitamin B_{12}, excreted more urinary nitrogen so there was little or no increase in grams of nitrogen retained.

The lighter weight pigs on the lower levels of food intake, particularly the 2.9% level, exhibited a marked tendency toward a very low nitrogen retention value. These pigs of lighter weight were apparently absorbing less nitrogen per unit of metabolic size as they were consuming less nitrogen per unit of weight raised to the 0.734 power. Since pigs on the lower levels of food intake did not absorb a higher percentage of the ingested nitrogen, it could be expected that they would retain smaller amounts of nitrogen. It is also probable that the pigs on the lower level of feed intake were actually utilizing a part of the ingested protein to help meet their total energy requirements. The diet used also contained slightly less than 0.12% of tryptophan, which may have been slightly inadequate for light weight weanling pigs.

Highly significant differences ($P \leq 0.01$) resulted in grams of nitrogen retained per unit of metabolic size when the 12 subtreatments were considered. Differences in nitrogen retention due to level of feed intake were also significant. No significant difference existed in grams of nitrogen retained due

TABLE 1

Average nitrogen metabolism data for the pigs on the individual treatments [1]

TREATMENT	LEVEL OF FEED INTAKE, AS PER CENT OF BODY WEIGHT	MEAN BODY WEIGHT	AV. DAILY INCREASE	AIR DRY FEED CONSUMED DAILY	NITROGEN METABOLISM PER DAY				NITROGEN METABOLISM PER DAY PER $W_{kg}^{0.734}$		
					Intake	Feces	Urine	Balance	Absorbed	Urine	Balance
		kg	*kg*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm* [2]	*gm*	*gm*
1	2.8	39.9	0.43	1138	25.70	5.26	10.41	10.03	1.38	0.70	0.68
	3.5	43.5	0.71	1520	34.31	7.38	12.55	14.38	1.68	0.80	0.88
	4.0	47.7	0.61	1859	41.97	9.87	14.37	17.73	1.86	0.83	1.03
2	2.9 [3]	41.6	0.61	1188	26.83	5.58	10.23	11.02	1.36	0.67	0.69
	3.5	44.0	0.60	1542	34.80	6.19	14.79	13.82	1.75	0.91	0.84
	4.0	43.6	0.73	1724	38.92	8.44	15.77	14.71	1.89	0.97	0.92
3	2.9	39.5	0.40	1143	25.80	5.25	10.73	9.82	1.36	0.72	0.64
	3.5	42.5	0.64	1514	34.18	7.57	14.62	11.99	1.66	0.91	0.75
	4.1 [3]	42.6	0.61	1716	38.73	8.50	14.76	15.47	1.90	0.94	0.96
4	2.9	43.7	0.47	1279	28.88	5.05	13.40	10.43	1.46	0.82	0.64
	3.5	44.4	0.65	1575	35.56	6.48	15.05	14.03	1.76	0.94	0.82
	4.2	45.7	0.85	1924	43.43	7.74	17.54	18.15	2.12	1.04	1.08

[1] Each figure represents the average of 6 collections.

[2] Apparently absorbed nitrogen.

[3] Average data for only the 5 pigs on which total data were obtained.

to treatment and within feed intake levels. Thus, neither chlortetracycline nor vitamin B_{12}, nor a combination of the two, exerted a sparing action on the protein requirement of the pig as measured by the nitrogen balance technique.

The adjusted mean nitrogen retention figures for each of the 12 possible subtreatments are shown in table 2. These adjusted values were obtained by application of the within treatment regression coefficient of 0.03 per unit of metabolic

TABLE 2

Adjusted mean nitrogen values, grams per $W_{kg}^{0.734}$.

TREATMENT	LEVEL OF FEED INTAKE	MEAN $W_{kg}^{0.734}$	DEVIATION FROM MEAN	CORRECTION [1]	MEAN NITROGEN BALANCE	ADJUSTED MEAN NITROGEN BALANCE [2]
					gm	*gm*
1	2.9	14.57	— 1.08	— 0.03	0.68	0.70
	3.5	15.74	0.10	0.00	0.87	0.87
	4.0	16.82	1.18	0.03	1.03	1.00
2	2.9	15.29	0.36	0.01	0.74	0.73
	3.5	15.90	0.25	0.01	0.84	0.83
	4.0	15.71	0.06	0.00	0.92	0.92
3	2.9	14.74	— 0.90	— 0.03	0.65	0.68
	3.5	15.38	— 0.27	— 0.01	0.75	0.76
	4.1	15.47	— 0.17	— 0.00	1.00	1.00
4	2.9	15.81	0.16	0.00	0.64	0.64
	3.5	15.99	0.34	0.01	0.82	0.81
	4.2	16.33	0.68	0.02	1.08	1.06

[1] Deviation multiplied by the error regression coefficient (0.03).

[2] Mean nitrogen retention minus correction.

size. The regression equation for these calculations is $Y = 0.03X + 0.39$ where Y equals the adjusted mean nitrogen retention value in grams per metabolic unit and X equals the mean metabolic size value, 15.64. Adjusted mean nitrogen retention values in grams per metabolic unit, ignoring treatments, were 0.69, 0.82 and 0.99, respectively, for pigs fed at feed intake levels of 2.9, 3.5 and 4.1% of body weight.

When the increase in nitrogen retention reported by Russo et al. (54) is considered it appears that even the 14.1% crude

protein diet used in this trial may not have been sufficiently limiting in total nitrogen, particularly for the heavier pigs, to produce results which would indicate increased nitrogen retention due to chlortetracycline, vitamin B_{12}, or a combination of the two. The level of feed intake used by these investigators is not known. The study reported here was intentionally conducted with a diet containing 14.1% of crude protein, a level of protein believed to be adequate but considerably below that recommended by the National Research Council ('53).

SUMMARY AND CONCLUSIONS

Growing barrow pigs weighing from 19.50 to 77.44 kg (mean 43.50 kg) and with metabolic size values of 8.49 to 23.35 (mean 15.64) did not show a significant increase in nitrogen retention when a 14.1% crude protein diet was supplemented with either 22 mg of chlortetracycline, 11 μg of vitamin B_{12}, or a combination of the two, per kilogram of diet.

Highly significant correlations were found between body weight in kilograms raised to the power 0.734 and nitrogen retention within each of the three levels of feed intake used. Highly significant differences in nitrogen retention were demonstrated to exist due to level of feed intake.

Pigs consuming more total feed were able to utilize their nitrogen more efficiently for body gains as evidenced by the highly significant increases in nitrogen retention due to increased level of feed intake.

Neither chlortetracycline nor vitamin B_{12}, nor a combination of the two, exerted a sparing action *per se* upon the protein requirement of growing swine fed the 14.1% protein diet used in this study.

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THE RELATION OF SERUM CHOLESTEROL TO THE PHYSICAL MEASUREMENTS AND DIET OF WOMEN[1]

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A higher incidence of coronary disease and hypertension was observed in the obese (Masters et al., '53). Although it is by no means certain that small differences in the blood cholesterol levels in man influence atherogenesis, hypercholesterolemia appears to be associated with obesity (Gofman and Jones, '52) and cardiovascular atherosclerosis (Gertler et al., '50; Steiner et al., '52; Soffer and Murray, '54). Because most of these observations have been based upon studies of men, an investigation of the serum cholesterol of women in relation to the diet and physical measurements was considered worth while.

The purpose of the present inquiry was to investigate the relationship of varying levels of dietary fat, protein and energy intakes to the serum cholesterol as well as to study its association with the diastolic blood pressure, age and body weight. Additional data of serum cholesterol in healthy women are needed in order to establish values for various age groups.

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METHODS

Volunteers for this study were 113 homemaker and professional women from 26 to 92 years of age living in their homes. All were in apparent good health. Each subject was examined clinically by the university physician who also recorded the age, blood pressure, height and weight. A measured, self-selected, 7-day dietary intake was recorded by the subject prior to obtaining the blood sample. The fat, protein and total energy intakes were computed from the tables of Bowes and Church ('52). As an index of over- and underweight the relative body weight in pounds per inch of height was used.

Blood was obtained one-half to three hours after a light breakfast, except for 17 subjects from whom it was removed after a small evening meal. The blood cholesterol data from all subjects were pooled. The data were pooled since diurnal fluctuations (Chandler et al., '53) and basal compared with non-basal variations (Keys et al., '50) of the serum cholesterol have been shown not to be significant. Even a high-fat meal did not alter the serum cholesterol (Pomeranze et al., '54) during the following 24-hour period. Blood was obtained by venipuncture, allowed to clot under refrigeration, and then centrifuged. Serum was immediately removed and stored at — 4°C. from 4 to 22 weeks. The free and total cholesterol were determined by the method of Sperry and Webb ('50).

Since serum samples were thawed several times while stored for varying periods, an investigation was undertaken to study these effects on the free and total serum cholesterol. The analysis of 6 fresh serum samples was compared with that of the same samples after 4 and 22 weeks of storage. During storage the serum was thawed once, twice or three times at intervals of 4, 6 and 22 weeks, respectively.

RESULTS AND DISCUSSION

Storage. The length of the storage period as well as the number of thawings had no significant effect upon the total

and free serum cholesterol concentrations when compared with fresh serum samples. The average total and free cholesterol values plus and minus the standard deviations in milligrams percent were respectively: (a) 193 ± 34.2 and 50.0 ± 9.41 for fresh serum, (b) 190 ± 37.9 and 48.0 ± 10.5 after 4 weeks of storage and (c) 192 ± 33.5 and 45.2 ± 10.1; 197 ± 39.1 and 47.2 ± 9.11; and 196 ± 31.8 and 45.0 ± 10.2 for samples stored 22 weeks and thawed once, twice and thrice, respectively.

Serum cholesterol for various age groups of women. Mean values for the total and free serum cholesterol and the ratio of the free to the total cholesterol for all subjects by decades

TABLE 1

Total and free serum cholesterol for various age groups of women

NO. OF SUBJECTS	AGE			SERUM CHOLESTEROL					
				Total		Free		Ratio of free/total	
	Range	Mean	S.D.[1]	Mean	S.D.	Mean	S.D.	Mean	S.D.
		yrs.		*mg %*		*mg %*			
6	< 30	27.3	1.11	207	53.1	51.0	20.3	0.239	0.041
36	30–39	34.5	2.81	211	37.6	53.5	11.1	0.253	0.017
29	40–49	44.6	3.66	250	66.6	64.3	18.9	0.256	0.018
13	50–59	54.2	3.20	293	57.7	78.1	16.4	0.266	0.011
20	60–69	65.8	2.10	302	63.9	79.5	18.5	0.262	0.013
9	⩾ 70	77.6	8.85	239	41.1	62.2	10.6	0.261	0.015
113	26–92	48.1	14.9	249	64.3	64.3	18.8	0.256	0.019

[1] The standard deviation of the distribution.

is given in table 1. The ratio of the free to the total serum cholesterol was constant for all age groups. A mean total serum cholesterol value of 249 ± 64.3 mg % was found for 113 women from 26 to 92 years of age. The values range from 141 to 502 mg %.

With increasing age the total serum cholesterol is observed to gradually increase (fig. 1). A significant rise ($p < 0.01$) from 207 ± 53.1 to 293 ± 57.7 mg % at 27.3 and 54.2 years of age respectively is evident. The latter high cholesterol level is maintained until 65.8 years of age. Beyond this age a significant decline ($p < 0.01$) to 239 ± 41.1 mg % at 77.6 years of age occurs.

The cholesterol-age trend noted in the present investigation concurs with the findings of others (Gillum et al., '55; Swanson et al., '55; Kountz et al., '45) who observed that a maximum cholesterol value in women usually occurred between 60 and 70 years of age. Our data demonstrated the maximum

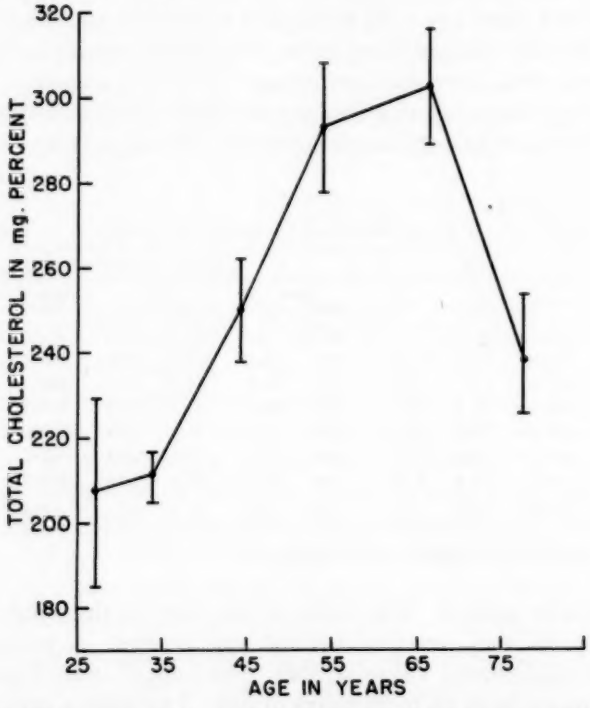

Fig. 1 The average total serum cholesterol values for women grouped by decades. The vertical line represents plus and minus one standard error, the center of which is the average.

occurred a few years earlier in women. This peak was evinced at approximately 45 to 55 years of age in men (Keys et al., '50; Gillum et al., '55).

Although it is well known that women have higher cholesterol values than men, a wide range of total serum cholesterol

values has been recorded in the literature for American women. Swanson et al. ('55) studying a similar age group of women found a significantly lower average serum cholesterol concentration. They reported a mean value of 209 mg % of blood cholesterol for 184 women with an average age of 48 years. For women 50 or more years of age our value of 285 mg % of total cholesterol is comparable to that of 270 mg % reported by Gillum and co-workers ('55) for 246 women. A lower mean total serum cholesterol value of 237 mg % for women over 40 years of age (Kountz et al., '45) as well as a higher mean value of 310 mg % for women beyond 61 years of age (Hobson et al., '53) has been reported than is found in comparable age groups of women in the present study who had a mean of 322 and 283 mg % respectively.

Correlations of the physical measurements with the serum cholesterol. Since hypercholesterolemia was found in the obese men (Gofman and Jones, '52) and the hypertensive (Stewart and Basu, '51), the tenability of these findings as well as the relationship of serum cholesterol to age was investigated in women.

TABLE 2

Serum cholesterol levels associated with age, relative body weight and diastolic blood pressure

NO. OF SUBJECTS	TOTAL SERUM CHOLESTEROL			AGE		RELATIVE BODY WEIGHT		DIASTOLIC BLOOD PRESSURE	
	Range	Mean	S.D.[1]	Mean	S.D.	Mean	S.D.	Mean	S.D.
	mg %	*mg %*		*yrs.*		*lbs./in.*		*mm of Hg*	
17	< 160–189	166	14.7	36.8	11.1	2.02	0.408	78	20.5
20	190–219	203	7.79	41.1	11.0	2.08	0.246	71	9.78
26	220–249	234	8.86	47.0	15.0	2.20	0.303	80	16.2
21	250–279	262	7.19	51.2	15.0	2.25	0.433	82	14.5
12	280–309	293	5.71	56.9	14.4	2.56	0.364	90	13.4
9	310–339	323	10.3	60.3	8.27	2.36	0.538	80	10.3
3	340–369	354	11.0	58.0	8.60	2.45	0.318	108	8.64
5	≥ 370	431	60.4	57.4	8.52	2.18	0.373	93	14.6
113	142–502	249	64.3	48.1	14.9	2.22	0.403	81	16.5

[1] The standard deviation of the distribution.

Mean values for age, relative body weight and diastolic blood pressure which correspond to the increasing levels of serum cholesterol by 30 mg % intervals are given in table 2. All variants show curvilinear variations with rectilinear increases of the serum cholesterol values.

An equation was fitted by the method of least squares using weighted groups that best described the variations of age, relative body weight and diastolic blood pressure with the logarithm of the total serum cholesterol as follows:

$$\log x_1 = -2.427 - 0.000256x_2 + 0.507x_3 - 0.0577x_4^2 + 2.085 \log x_4$$

where

x_1 = total serum cholesterol
x_2 = age
x_3 = relative body weight
x_4 = diastolic blood pressure

The logarithm rather than the natural number for the value of cholesterol was used since the chi square test indicated a more normal distribution. All regression coefficients for the above equation were significant ($p < 0.01$).

Multi-variable analysis of the individual data using the digital computer gave a multiple correlation coefficient of 0.559 (probability of significance, $p = 0.01$) indicating that 31% ($R^2 = 0.309$) of the variation in serum cholesterol is positively associated with age, relative body weight and diastolic blood pressure. The partial correlation coefficients were 0.423 ($p < 0.01$) relative to age alone, 0.210 ($p = 0.05$) with respect to the relative body weight alone and 0.144 (not significant) in relation to the diastolic blood pressure alone. Approximately 18% ($r^2 = 0.179$) of the variation in serum cholesterol is significantly associated with age and 4% ($r^2 = 0.044$) with the relative body weight, whereas, a negligible variation is attributable to the diastolic blood pressure.

Although most of the variation in serum cholesterol is associated with age, the interpretation of this finding is a matter of conjecture. The rise and decline of serum cholesterol from youth to old age may represent in each successive age group an association with those survivors who are not typical of younger population groups. Swanson and co-workers ('55) using a multiple linear regression also noted that age ac-

counted for almost all of the variation in serum cholesterol.

Similar to our findings a positive correlation between the relative body weight and skinfold thickness with hypercholesterolemia, independent of age, was found in a population group of Spanish women by Keys and co-workers ('54). Hobson et al. ('53) also reported this relationship in American women using the skinfold thickness. Neither of these groups of workers, however, was able to demonstrate this positive correlation in men. Yet, Walker ('53) observed in both sexes that overweight was positively associated with elevated serum cholesterol levels. Negligible correlations between body weight and serum cholesterol values for supposedly normal women were reported by Gillum et al. ('55) and Swanson et al. ('55).

Surveys of population groups heretofore have not demonstrated a relationship between the systolic or diastolic blood pressure (Hobson et al., '53; Gillum et al., '55; Swanson et al., '55) with the serum cholesterol.

Correlations of the diet with the serum cholesterol. Controlled human experiments have established that a marked increase or decrease in the level of dietary fat causes a corresponding rise or fall of the serum cholesterol (Hildreth et al., '51; Mayer et al., '54; Anderson and Keys, '53). Negative caloric balances without fat restriction inducing weight loss (Pomeranze et al., '54; Walker et al., '53) revealed a significant decrease of serum cholesterol only in those overweight persons with previously high cholesterol values. However, rapid weight gain induced by a high caloric, fat-free diet resulted in a marked rise of initially low serum cholesterol values (Walker et al., '53). Because changes in the serum cholesterol levels appeared to be related to the fat intake as well as to the total calories, the present study investigated by multi-variable analysis the relationship of the total serum cholesterol with the total energy, dietary fat and protein intake. The latter factor was included since protein is known to contain the lipotropic substance methionine.

The percentage of fat and protein calories and total energy for all subjects by 30 mg % intervals of serum cholesterol

is presented in table 3. These three variants demonstrated curvilinear variations with respect to the serum cholesterol. A multiple correlation value of 0.199 revealed that the variation in serum cholesterol is not significantly associated with the percentage fat and protein calories and total energy.

Nutritional status investigations of Wilkinson and coworkers ('50) and Swanson et al. ('55) did not find a relationship between the serum cholesterol and the fat and protein intakes. But other surveys of aging persons by Hobson et al.

TABLE 3

Serum cholesterol levels associated with the per cent fat and protein calories and total energy intake

NO. OF SUBJECTS	TOTAL SERUM CHOLESTEROL			% FAT		% PROTEIN		TOTAL ENERGY	
	Range	Mean	S.D.[1]	Mean	S.D.	Mean	S.D.	Mean	S.D.
	mg %	*mg %*		*Cal.*		*Cal.*		*Cal.*	
17	160–189	166	14.7	38.7	4.45	16.4	1.74	1958	443.8
20	190–219	203	7.79	39.8	4.75	15.4	2.09	2044	372.0
26	220–249	234	8.86	37.5	4.80	15.6	1.47	1970	298.6
21	250–279	262	7.19	40.7	4.67	16.4	1.90	1922	314.6
12	280–309	293	5.71	39.8	5.18	16.1	1.92	1855	367.7
9	310–339	323	10.3	39.9	5.67	16.0	2.62	1897	247.5
3	340–369	354	11.0	43.0	5.28	15.9	1.94	1946	181.6
5	≧370	431	60.4	40.5	4.14	15.3	1.03	2100	305.6
113	142–502	249	64.3	39.4	4.99	15.9	1.91	1959	349.1

[1] The standard deviation of the distribution.

('53) showed a low but significant positive correlation between the fat consumption and the serum cholesterol in women but not in men, whereas that by Gillum et al. ('55) observed this relationship in both sexes. The latter group also reported a significant positive correlation of the protein intake with the serum cholesterol for men but not for women.

This and other nutritional status studies indicate that dietary surveys and physical measurements in conjunction with determinations of blood cholesterol have not consistently yielded relationships compatible with those findings established by controlled human dietary experiments.

SUMMARY

Storage of frozen serum samples for periods of 4 and 22 weeks and thawing of these samples as often as three times did not alter the concentrations of the free and total serum cholesterol when compared with the values in fresh samples.

A mean total serum cholesterol concentration of 249 mg % was observed for 113 women from 26 to 92 years of age. The average cholesterol content of the serum increased gradually from 207 mg % to a maximum of 293 mg % at a mean age of 27.3 and 54.2 years, respectively. This maximum value was maintained during the 6th and 7th decades of life. Thereafter, the concentration of cholesterol declined to 239 mg % at 77.6 years of age. The ratio of the free to total cholesterol was constant for all age groups.

Significant positive correlations for age and relative body weight but a negligible correlation for the diastolic blood pressure with the total serum cholesterol was found. The percentage of dietary fat and protein calories as well as the total energy intake did not show a significant relationship with the variation in total blood cholesterol.

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THE EFFECT OF RADIATION STERILIZATION ON THE NUTRITIVE VALUE OF FOODS

I. BIOLOGICAL VALUE OF MILK AND BEEF PROTEINS [1]

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During the past few years considerable interest has been shown regarding the use of ionizing radiations for processing and preservation of foods. Radiant energy at high dosages will not only destroy all microorganisms by inducing changes in their chemical structure but will also bring about chemical changes in the food. These changes may affect the acceptability as well as the nutritive value of the food. Before a new food processing method can be accepted evidence is necessary demonstrating that little or no deleterious change takes place in the nutritive value of the food constituents. Proctor and Bhatia ('50) have reported that the 10 essential amino acids of haddock fillets are not significantly destroyed upon irradiation sterilization. However, the same investigators ('53) have reported that when aqueous solutions of amino acids were exposed to high-voltage cathode rays deamination resulted and the benzene rings in tryptophan, phenylalanine and tyrosine were broken. We have undertaken experiments to investigate the effect of irradiation sterilization of foods on the nutritive value of their proteins and the availability of their energy for the rat. In general the data indicate that

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ionizing radiations have little or no effect on the biological values of the proteins nor on the metabolizable energy of the foods tested.

The purpose of this paper is to report (1) the changes in the protein value of beef round brought about by irradiation sterilization;[2] and (2) the changes in the nutritive value of the milk proteins brought about by subjecting "evaporated[3] milk" to heat sterilization[4] and irradiation sterilization, respectively.

Our data demonstrate that irradiation sterilization like heat sterilization does not affect the digestibility or the biological value of beef protein or milk protein. The biological value of milk protein was reduced by 8% by irradiation as compared to a reduction of 6% resulting from heat sterilization of milk. The biological value of beef protein was unaffected by irradiation.

EXPERIMENTAL PROCEDURE

The effect of heat and irradiation on beef and milk were tested with regard to the nutritional value of their conventional protein ($N \times 6.25$) by nitrogen metabolism studies with male albino rats in the early phase of growth. Mitchell's method of measuring the nutritive value of a protein (Mitchell, '24, '44) which permits the computation of coefficients of true digestibility, making due allowance for the metabolic fecal nitrogen, and of biological value (the percentage of the absorbed nitrogen used for the replacement of endogenous

[2] Irradiation was carried out by Phillip's Petroleum Company, Arco, Idaho. A 3 million rep (roentgen equivalent physical, 93 ergs of radiation energy absorbed per gram) gamma irradiation was used to "sterilize" the beef and milk samples.

[3] The "evaporated" milk received the following treatment: prior to condensing, the fresh milk was held at 210°F. for 10 minutes preceeded by a 20-minute temperature rise period during which the milk was brought to temperature. The milk was condensed in a double effect evaporator and remained in the first effect for approximately 2½ minutes at 180°F. and in the second effect for approximately 2½ minutes at 125°F.

[4] The heat-sterilized milk was treated as was the evaporated milk and in addition a sterilization treatment was carried out in an Anderson-Barngrover Continuous Sterilizer at 242°F. for 14 minutes.

losses and for growth) was followed. In all, three experiments were carried out. The first two experiments undertaken on different lots of milk on separate occasions compared the effects of heat and irradiation, respectively, on the nutritive value of the proteins of non-sterilized evaporated milk. The third experiment compared the nutritive value of the proteins of raw and irradiated beef. In each experiment 7 growing rats of the Sprague-Dawley strain were used per treatment. There were three experimental feeding periods in the first test, but only two such periods in the second and the third tests. The experimental diets were fed in periods 1 and 3, while in period 2 all rats received the standardizing diet containing 4% of

TABLE 1

Plan of feeding in experiment 1

PERIOD	GROUP I	GROUP II	GROUP III
1	Non-processed milk	Heat-processed milk	Radiation-sterilized milk
2	4% whole egg diet	4% whole egg diet	4% whole egg diet
3	Radiation-sterilized milk	Non-processed milk	Heat-processesd milk

whole egg protein. During this second period the ratio of metabolic fecal nitrogen to food consumed, and the ratio of endogenous urinary nitrogen to the three-fourths power of the body weight were determined. The plan of feeding in the first experiment is given in table 1.

Each experimental feeding period lasted 16 days of which the first 9 days were allowed for physiological adjustment to the test diets and during the following 7 days urine and feces were collected. Ferric oxide was used as feces marker.

"Evaporated [5] milk" and heat-sterilized [6] evaporated milk samples were obtained from a commercial source.[7] Beef round steak was purchased from the University Meats Division. After trimming the visible fat, the beef was ground,

[5] See footnote 3, page 480.
[6] See footnote 4, page 480.
[7] Pet Milk Company, Greenville, Illinois.

canned under vacuum and frozen. Some of the cans of frozen "evaporated milk" and beef were irradiated with three million rep [8] gamma radiation. Proximate analyses of the non-processed and heat or irridation-processed beef and milk are given in table 2. The nitrogen contents of the raw beef and the irradiation sterilized beef on moisture free basis as measured by the Kjeldahl-Wilfarth-Gunning method were found to be 11.75 ± 0.061 (S.E.) and 11.77 ± 0.092 gm %, respectively. These two values were not significantly different ($P = 0.7$). So also the nitrogen content, on dry basis, of evap-

TABLE 2

Chemical analysis of milk and beef samples

CONSTITUENT	RAW EVAPORATED MILK	HEAT STERILIZED EVAPORATED MILK	IRRADIATION STERILIZED EVAPORATED MILK	RAW BEEF	IRRADIATION STERILIZED BEEF
	%	%	%	%	%
Moisture	74.7	74.3	74.1	69.3	68.2
Ether extract	...	...	...	7.3	6.6
Total nitrogen	1.118	1.117	1.120	3.60	3.74
Crude protein (N × 6.25)	6.90	6.98	6.90	22.5	23.4

orated milk (4.42 ± 0.09 gm %) was not significantly different ($P = 0.5$) from that of the irradiated milk (4.33 ± 0.11 gm %). These data on total nitrogen show that little or no deamination of the amino acids from the intact proteins had taken place upon irradiation. This agrees with the report of Proctor and Bhatia ('50) who found no change in the amino acid composition of the haddock fillets when irradiated.

Milk and beef samples were incorporated fresh into experimental rations to provide 10% of protein, respectively, on a moisture-free basis. The composition and proximate analysis of the test diets are provided in tables 3 and 4. The test rations were preserved in air-tight feed jars kept in a deep

[8] See footnote 2, page 480.

freeze room (0°F.). The feed jars, one of each diet, were removed from the deep freeze every third day. The jars so withdrawn were stored in a cold room at 40°F., and the diets from these jars were fed to the animals for a period of three days. Left-over diets after three days were discarded, and

TABLE 3

Composition of rations

CONSTITUENT	EVAPORATED MILK DIET	HEAT STERILIZED MILK DIET	IRRADIATION STERILIZED MILK DIET	RAW BEEF DIET	IRRADIATION STERILIZED BEEF DIET
	gm	*gm*	*gm*	*gm*	*gm*
"Evaporated milk"	36.8 [1]	...	...	...	...
Heat-sterilized evaporated milk	...	36.8 [1]	...	...	...
Irradiation sterilized evaporated milk	...	...	36.8 [1]	...	...
Raw beef	...	...	...	13.6 [1]	...
Irradiation sterilized beef	...	...	...	...	13.6 [1]
Cornstarch	39.7	39.7	39.7	30.0	30.0
Cerelose	...	...	...	10.0	10.0
Sucrose	10.0	10.0	10.0	10.0	10.0
Modified cornstarch [2]	...	...	...	17.8	18.0
Lard	...	...	...	5.1	4.9
Vitaminized cerelose [3]	5.0	5.0	5.0	5.0	5.0
Cod liver oil	1.5	1.5	1.5	1.5	1.5
Wheat germ oil [4]	0.5	0.5	0.5	0.5	0.5
Salts 446 [5]	4.0	4.0	4.0	4.0	4.0
Sodium chloride	0.5	0.5	0.5	0.5	0.5
Wood Flock [6]	2.0	2.0	2.0	2.0	2.0
Total	100.0	100.0	100.0	100.0	100.0

[1] On moisture-free basis.

[2] Amidex.

[3] The composition of vitaminized cerelose is as follows: calcium pantothenate 40 mg; pyridoxine hydrochloride, riboflavin and thiamine hydrochloride 5 mg each; nicotinic acid 20 mg; p-aminobenzoic acid 100 mg; inositol 200 mg; choline chloride 2 gm; menadione 2 mg; and cerelose 97.6 gm.

[4] Viobin.

[5] Specter ('48).

[6] Distributed by Brown Company, Portland, Maine.

freshly withdrawn diets from the deep freeze were used. This method of storing the diets and feeding the animals was followed because the diets were found not to keep at 40°F. for more than 4 to 5 days.

TABLE 4

Chemical analysis of experimental diets

DIET	MOISTURE	ETHER EXTRACT	CRUDE PROTEIN (N × 6.25)	GROSS ENERGY
	%		%	*Cal.*
"Evaporated milk"	52.51	..	4.9	209
Heat sterilized milk	53.12	..	5.0	210
Irradiation sterilized milk	53.67	..	5.0	210
Raw beef	28.3	6.4	8.24	343
Irradiation sterilized beef	27.4	6.5	8.26	342

RESULTS AND DISCUSSION

The effect of heat sterilization and of irradiation sterilization on milk proteins

When the milk cans from the three different treatments were thawed and opened, the non-processed milk looked and tasted just like fresh evaporated milk; the heat-sterilized looked and tasted exactly like commercial evaporated milk. The irradiation-sterilized milk, on the other hand, was somewhat darker in color with a slight reddish tinge, and the proteins had been denatured and coagulated by the irradiation treatment so that the milk no longer was an homogeneous solution but a suspension of particles. This milk tasted slightly more "burnt" than did the heat-sterilized milk.

In both experiments the rats receiving the raw milk diet and the heat sterilized milk diet consumed the rations quickly within one-half to three hours, whereas the rats given the irradiation sterilized milk diet ate reluctantly taking 8 to 18 hrs. Slight diarrhea was also noticed in rats receiving irradiation-sterilized milk. However, all rats grew at the rate of about 2 gm a day which may be considered satisfactory for a biological value test (Forbes and Yohe, '55). The data

of the biological value tests were treated statistically according to the analysis of variance and the results are summarized in table 5.

Apparently the process of evaporation [9] of the raw milk has not altered the biological value of the milk proteins since the biological value of 89.5 for the proteins of the evaporated milk which we obtained compares very well with the value of 89.8 reported by Fairbanks and Mitchell ('35) for raw liquid skim milk. Pasteurization of milk is generally done by holding

TABLE 5

Average coefficients of apparent and true digestibility and biological values of protein for the various milk samples

EXPERIMENT NO.	LIQUID "EVAPORATED" MILK	HEAT STERILIZED EVAPORATED MILK	RADIATION-STERILIZED EVAPORATED MILK	STANDARD ERROR
		Apparent digestibility, %		
1 and 2	86.4	85.2	85.3	0.39
		True digestibility, %		
1 and 2	97.9	96.7	96.9	0.46
		Biological value, %		
1, Period I	92.0	88.2	85.5	0.89
1, Period III	89.1	80.5	77.4	0.89
2	87.2	84.1	82.5	0.97
1 and 2	(89.5)	84.3	81.8	0.53

milk at 143°F. for 30 minutes. Henry and Kon ('36), Krauss ('37), Bixby et al. ('54) and many others have reported data which indicate that pasteurization has little or no effect on the nutritive value of milk proteins. The "evaporated milk," therefore, is as nutritious as raw milk or pasteurized milk as far as the milk proteins are concerned.

The digestibility of milk proteins. Detailed statistical analysis of experiment 1 found no appreciable difference between periods or between trios or between rats within trios; these differences were therefore pooled with error. Comparison of error mean squares for the two experiments found them to

[9] See footnote 3, page 480.

be in good agreement, justifying a combined analysis. The analysis showed that the two experiments agreed as well as they should as to the magnitude of the treatment effects. The apparent digestibility of "evaporated milk" differed significantly from the average of the processed milk at the 2% level and there was an average difference between experiments at the 2% level. However, the true digestibility of "evaporated milk" did not differ from the average of the heat-sterilized or radiation-sterilized milk ($P = 0.05$), and there was no difference between experiments ($P = 0.05$).

Kon and Henry ('38) reported a slight but not significant difference in the true digestibility of the proteins of the raw and heat-sterilized milk. Their average values were 96.4 and 95.3% for raw and heat-sterilized milk, respectively, which compare well with our values of 97.9% for the raw milk, 96.7% for heat-sterilized and 96.9% for radiation-processed milk proteins. Fairbanks and Mitchell ('35) also found only slight difference in true digestibility of the proteins of raw milk and of choice commercial roller-dried or spray-dried preheated milk powder.

Biological value of the milk proteins. Statistical analysis of biological values found significant differences ($P < 0.01$) in experiment 1 between periods I and III and between trios but not between rats within trios. A significant difference ($P < 0.01$) between trios was also found in experiment 2. In experiment 1 there was a significant fluctuation (< 0.01) of treatment effects from period I to period III. Experiments 1 and 2 agreed well as to the magnitude of the experimental error, and a combined analysis was performed. There was a significant difference ($P < 0.001$) between treatments, chiefly "evaporated milk" versus treated milk but also heated versus irradiation-sterilized milk, and the difference between "evaporated" and treated fluctuated between the two experiments almost enough to reach the 1% significance level, and between the two periods of experiment 1 enough to pass the 1% level.

Since radiation and heat processing of milk do not affect the digestibility but do affect the biological value of its pro-

tein this means most probably that some essential amino acid has been partially destroyed or bound so as not to be available in the animal and that this amino acid is or has become the limiting amino acid of this treated protein. It may be that due to binding of some sort (e.g. cross link formation) some amino acids are not being released at an optimum rate, causing the amino acid pool to be less efficiently utilized, or are being released in the lower part of the intestine where they undergo alteration due to the flora and, though absorbed, are not available for growth (Carroll et al., '51). Hodson ('54) reported that heat sterilization slightly but significantly lowers the growth-promotive value of evaporated milk and that supplementation with methionine, cystine, and cysteine almost restores the loss of value. Fairbanks and Mitchell ('35) concluded that the proteins of milk are very sensitive to heat treatment. They found that the biological values were lowered by 8%, although the digestibility was not affected when moderate heat processing was used for drying the milk. Supplementation of such dried milk with cystine restored its nutritive value. However, when the temperature of drying in the roller process was increased until perceptible scorching occurred, they observed a further decrease of 12% in the biological value. They state: "the scorched products thus obtained are no longer benefited by cystine additions, but they do respond to lysine additions in increased nutritive value of their proteins." Hence the rapid change in milk proteins at the scorching point (or earlier) is primarily a result of the destruction of lysine. Proctor and Bhatia ('53) measured the ammonia yields from 0.1 M solutions of some amino acids upon irradiation with a cathode-ray dose of 250,000 rep and found that the order of deamination of the amino acids studied was: histidine > cystine > phenylalanine > tyrosine > tryptophan. These investigators ('52) had also observed evolution of hydrogen sulfide upon irradiation of aqueous solutions of cystine thereby indicating that the molecule of cystine was decomposed at the disulfide linkage.

The effect of irradiation sterilization on beef proteins

Upon opening the cans the irradiated beef looked somewhat darkened and had a slight acrid odor. However, these changes were not pronounced, and the irradiated beef diet was completely acceptable to the rats during the entire feeding period. The group fed the irradiation-sterilized beef diet ate as avidly as the group receiving the raw beef diet. No food refusals were observed at any time.

TABLE 6

Average coefficients of digestibility and biological values of the proteins of raw and irradiation sterilized beef

(Means of 9 observations with standard errors)

BEEF TESTED	APPARENT DIGESTIBILITY	TRUE DIGESTIBILITY	NITROGEN BALANCE	BIOLOGICAL VALUE
	%	%	*mg N/gm N intake*	%
Raw	91.8 ± 0.08	100 ± 0	579 ± 10	78.0 ± 1.0
Irradiated	92.3 ± 0.50	100 ± 0	592 ± 21	78.6 ± 1.5
Probability	0.4	1.0	0.5	0.7

While the rats were on the standardizing (low-nitrogen) diet the mean value for the metabolic nitrogen in the feces was found to be 2.18 ± 0.035 (S.E.) mg/gm of (moisture-free) food intake and for the endogenous nitrogen in the urine, 0.49 ± 0.019 mg/gm of body weight$^{3/4}$. The values agree well with similar values reported in earlier publications from this laboratory.

Statistical analysis of the nitrogen metabolism data reveals that there is no significant difference in the digestibility (apparent or true) or biological value of the proteins of the raw and the radiation-sterilized beef (table 6). For the raw and irradiation-sterilized beef the apparent digestibility figures are 91.8 and 92.3%, respectively, for the true digestibility, 100 and 100%, respectively, and for biological value, 78.0 and 78.6%, respectively. These values compare very well with those of Mitchell and coworkers ('49), who have reported results of a study of the biological values of raw and roast beef.

These investigators found no significant difference in the digestibility or biological values, the percent true digestibility of the raw beef nitrogen being 99.6 and that of the roast beef, 99.3, and the percent biological values of the raw and roast beef proteins being 73.8 and 74.8, respectively.

SUMMARY AND CONCLUSIONS

The effect of irradiation sterilization and of heat sterilization on the nutritive value of milk proteins and beef proteins has been studied by the Thomas-Mitchell method. A 3 million rep gamma irradiation was used to sterilize the frozen milk and beef. The ground beef was vacuum packed during irradiation treatment and milk was conventionally canned. Upon irradiation milk developed a reddish tinge, and the proteins had coagulated; the beef looked somewhat darkened. Irradiation did not produce pronounced off-flavors and odors in beef or milk. The irradiated beef when incorporated into a balanced diet was found completely acceptable to the rat while the diet containing irradiated milk was consumed reluctantly.

Irradiation sterilization did not affect the apparent or true digestibility of the beef (100%) or of the milk protein (98%). The biological value of the beef protein (78%) also was not affected upon irradiation but the biological value of the milk proteins (90%) was reduced by 8% upon irradiation as compared to a reduction of 6% due to heat sterilization. The possibility of irradiation damage to the sulfur amino acids is discussed.

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THE INFLUENCE OF CREATINE BIOSYNTHESIS ON THE ARGININE REQUIREMENT OF THE CHICK[1]

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Arginine has been established as an essential amino acid for the chick (Klose et al., '38). Working with 4-week-old chicks, Almquist and Merritt ('50) found the arginine requirement for optimum growth to be 1.2% of a semi-purified diet containing 20% protein. On the other hand, several recent investigations (Young et al., '53; Wietlake et al., '54; Snyder et al., '54) employing purified diets have demonstrated that this level of arginine is insufficient for the day-old chick. In an attempt to explain this discrepancy on the basis of dietary differences among workers, Griminger et al. ('55) examined the possibility of an arginine loss or non-availability in completely purified diets as a factor causing an apparent increase in the arginine requirement. This postulation, however, was not substantiated, for the arginine requirement was again shown to be significantly greater than 1.2% under conditions which prevented any possible loss of arginine from the diet.

It has been shown in this laboratory that muscle creatine, for which arginine serves as a precursor, increases linearly for the first 4 weeks of the chick's life (Fisher et al., '56). Wietlake et al. ('54) have also found that in day-old-chicks dietary creatine will exert a sparing effect on arginine. In

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view of these findings it seemed of interest to investigate the arginine requirement of the day-old chick with special reference to the influence of creatine biosynthesis on this requirement.

EXPERIMENTAL AND RESULTS

Male day-old chicks of a White Rock ♂ × New Hampshire ♀ cross were used in all trials. The chicks were raised in electrically heated batteries and supplied ad libitum with water and feed. The rations, with the exception of the chick starter mash used in experiment 1, were modifications of the basal diet shown in table 1. The basal diet contained 0.8% of arginine.[2] Creatine analyses were carried out on samples of leg muscle by the procedure of Rose et al. ('27).

Experiment 1. This experiment was conducted to establish more precisely the muscle creatine — age relationship of normally growing chicks. A group of birds was fed a standard starter ration and 5 birds were sacrificed at weekly intervals for creatine determination. Muscle creatine values are shown plotted against age in figure 1. It can be seen that the slope of the muscle creatine curve decreased sharply after 4 weeks. The muscle creatine content increased 63% between one day and 4 weeks of age whereas it increased only 10% between the 4th and 8th week. This finding suggests a difference in arginine requirement before and after 4 weeks.

Experiment 2. The purpose of this experiment was to establish the arginine requirement in the presence of dietary creatine. The design of the experiment, the weight gains, feed utilization and muscle creatine data are given in table 2. It can be seen that gains improved with increasing levels of arginine; above the 1.5% level, no further improvement was noted. Muscle creatine also increased with each additional increment of arginine, despite the presence of 0.8% of dietary creatine which alone suffices to give "normal" creatine values (Fisher et al., '56).

[2] Microbiological assay of casein samples yielded values of 3.9% arginine on a 16% nitrogen basis. The casein level of the diet was therefore adjusted on the basis of nitrogen content to give 0.8% total arginine.

TABLE 1

Composition of rations

BASAL DIET				CHICK STARTER	
Ingredients	Amount	Vitamins added	Amount	Ingredients	Amount
	%		*mg/kg*		*Per 100 lbs.*
Cerelose	61.06	Thiamine HCl	25	Conr meal — lb.	53.6
Casein (crude)	25.00 [1]	Riboflavin	16	Soybean meal (50%) — lb.	34.0
Salt mixture [2]	5.34	Calcium pantothenate	20	Alfalfa — lb.	3.0
Corn oil	3.00	Niacin	15	Corn distillers solubles — lb.	4.0
Non-nutritive fiber	3.00	Pyridoxine HCl	6	Butyl fermentation — lb.	1.0
Choline chloride	0.20	Biotin	0.6	B_{12}-antibiotic supplement — lb.	0.5
A & D (10,000 A–600 D_3)	0.10	Folic acid	4	Dicalcium phosphate — lb.	2.2
Glycine	2.00	Inositol	100	Mineral concentrate — lb.	1.0
DL-Methionine	0.30	Para-amino benzoic acid	2	Salt — lb.	0.5
Vitamins	+	Menadione	5	Choline chloride (25%)	96 gm
	100.00	α-Tocopherol acetate	20	Niacin	1 gm
		Ascorbic acid	250	A & D (1500 A–300 D_3)	0.2
		Vitamin B_{12}	0.02		

[1] N × 6.25 = 20.4%.

[2] For composition see Fisher et al. ('54).

Experiment 3. In view of the high arginine requirement for growth established in the previous experiment and the increasing muscle creatine with each increment of arginine despite the presence of 0.8% of dietary creatine, it seemed

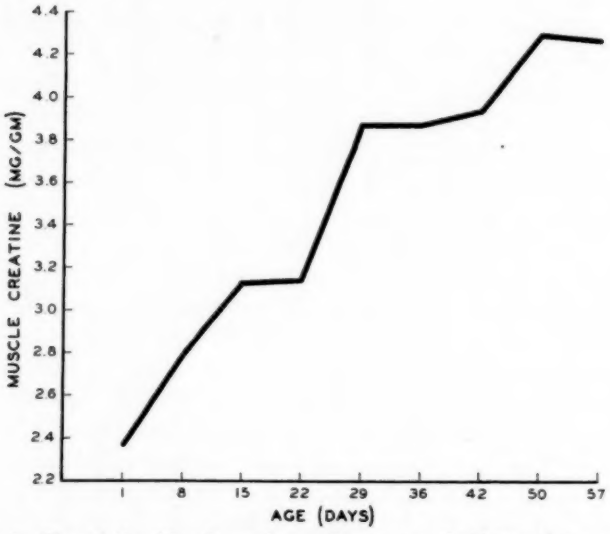

Fig. 1 The relationship of muscle creatine to age of chickens fed a practical starting ration.

TABLE 2

The arginine requirement of the chick in the presence of 0.8% dietary creatine hydrate

DIETARY LEVEL OF ARGININE	AVERAGE OF DUPLICATE GROUPS AT 3 WEEKS [1]		
	Gain	Gain/feed	Muscle creatine
%	*gm* *S.D.*[3]		*mg/gm* *S.D.*[3]
0.8 [2]	136 ± 15	0.445	3.59 ± 0.12
1.0	208 ± 14	0.584	3.65 ± 0.07
1.1	216 ± 11	0.604	3.65 ± 0.04
1.2	229 ± 9	0.658	3.78 ± 0.10
1.3	214 ± 8	0.647	3.82 ± 0.09
1.5	245 ± 10	0.639	3.96 ± 0.06
1.7	250 ± 8	0.632	3.99 ± 0.09

[1] Five birds per group.

[2] Amount present in basal diet.

[3] ± standard deviation of the mean.

necessary to ascertain what level of dietary creatine would yield optimum growth and muscle creatine in the absence of supplemental arginine (the basal diet contained approximately 0.8% of arginine). As shown in table 3, the levels of creatine ranging from 0 to 4% were fed, and weight gains, feed utilization and muscle creatine levels recorded at the end of three weeks. It can be seen that 1% of creatine was optimum in supporting growth and feed utilization on this diet. This, however, was not as good as could be obtained in the presence of adequate arginine. That 0.8% of arginine (present

TABLE 3

Effect of dietary creatine on growth and muscle creatine in the chick

DIETARY LEVEL OF CREATINE HYDRATE	AVERAGE OF DUPLICATE GROUPS AT 3 WEEKS [1]				
	Gain			Gain/feed	Muscle creatine
%	*gm*	*S.D.*[3]	*C.V.*[4]		*mg/gm* *S.D.*[3]
0 (basal) [2]	118 ± 21		(56)	0.448	2.63 ± 0.05
0.5	172 ± 17		(32)	0.532	3.42 ± 0.05
1.0	196 ± 13		(21)	0.609	3.59 ± 0.14
2.0	208 ± 12		(18)	0.586	3.75 ± 0.11
4.0	183 ± 14		(24)	0.517	4.11 ± 0.09

[1] Five birds per group.
[2] Containing 0.8% arginine.
[3] ± standard deviation of the mean.
[4] Coefficient of variation.

in the basal) was still limiting, even with 4% of supplemental creatine, was demonstrated by the upturned and frizzled feathers of all groups of birds which in our experience is characteristic of an arginine deficiency. The arginine deficiency is further reflected in the high coefficients of variation (table 3), an observation previously made in this and other laboratories (Wietlake et al., '54; Fisher et al., '56). Muscle creatine increased with every increment in dietary creatine. In view of the latter finding the results of experiment 2 can be interpreted to mean that some arginine is being diverted for creatine biosynthesis even when the muscle creatine content is already high or "normal." This would then explain

why the arginine requirement was still relatively high even in the presence of dietary creatine.

Experiment 4. This study was conducted to determine the arginine requirement of the chick in the presence and absence of dietary creatine. The experimental design and results are summarized in table 4. As in experiment 2, 1.5% of arginine supported optimum growth and efficiency in the presence of dietary creatine. In the absence of creatine, gains and feed

TABLE 4

The arginine requirement of the chick in the presence and absence of dietary creatine

DIETARY LEVEL OF		AVERAGE OF THREE REPLICATES AT 3 WEEKS [1]		
Arginine	Creatine hydrate	Gain	Gain/feed	Muscle creatine
%	%	*gm* *S.D.*[2]		*mg/gm* *S.D.*[2]
0.8	..	92 ± 9	0.387	2.80 ± 0.14
1.1	..	208 ± 14	0.520	3.16 ± 0.16
1.3	..	252 ± 10	0.575	3.24 ± 0.08
1.5	..	244 ± 10	0.593	3.24 ± 0.10
1.7	..	250 ± 8	0.615	3.58 ± 0.06
1.9	..	272 ± 10	0.657	3.65 ± 0.07
0.8	1.0	144 ± 12	0.489	3.56 ± 0.05
1.1	1.0	256 ± 8	0.604	3.68 ± 0.05
1.3	1.0	249 ± 10	0.614	3.68 ± 0.09
1.5	1.0	273 ± 8	0.637	3.70 ± 0.10
1.7	1.0	277 ± 6	0.653	3.81 ± 0.10
1.9	1.0	252 ± 9	0.645	3.84 ± 0.09

[1] Five birds per group.

[2] ± standard deviation of the mean.

efficiency continued to improve significantly with increasing levels of arginine. The supplementation with 1.9% of arginine gave the best results, with weight gains of the same order of magnitude as the maximum gain on the creatine-supplemented diet. It is interesting to note that the lowest muscle creatine value of the birds receiving the creatine-supplemented diet is comparable to the peak value of birds receiving only arginine.

DISCUSSION

An unusual feature of the data on arginine requirement is the growth plateau which occurs before the arginine level for maximum growth and feed efficiency has been reached. This plateau, which is *not* considered an artifact in view of its recurrence in each replicate and experiment, makes the statistical evaluation of a maximum response a difficult one. Regression analysis is complicated by the plateau and "t" test or least significant difference analyses are not necessarily apropos with dose-response data as pointed out recently by Almquist ('54).[3] Confirmation of the validity for this unusual growth plateau can be found in the arginine studies of Snyder ('54). He obtained the same plateau starting at the 1.3% arginine level, followed by a further increase in growth at higher arginine levels. He states the requirement to be 1.7%. This unique curve for arginine may be analogous to the lysine response curve of *S. fecalis* first obtained by Stokes et al. ('45).

The present data would indicate that the arginine requirement for the first three weeks of life on casein diets is greater than 1.3% and that for *maximum* growth and efficiency, dietary creatine will spare but a small amount of arginine.

The finding that some arginine is always being diverted for creatine synthesis parallels the observation by Fisher et

[3] "By the usual statistical tests it may be found that at the lower levels of F the response, $\triangle$ R, to a given $\triangle$ F is "significant." This significance is usually expressed as some ratio between the $\triangle$ R and the probable error, or standard error of $\triangle$ R, etc. As F increases, however, $\triangle$ R may become steadily smaller in reference to a given $\triangle$ F, according to the diminishing returns principle. It should be pointed out that this tendency alone may be sufficient to cause an application of the statistical tests for significance of difference to indicate that at higher levels of F observed, $\triangle$ R is no longer "significant." As a result, the investigators may conclude that there is no difference in R between two levels of F being tested near the top of the response curve. Thereupon, it may be decided that the lower level of F, of the two being compared, is as satisfactory as the higher level in meeting the full requirements of the animal. Because of this systematic error in interpretation, arising from disregard of the diminishing returns principle where it applies, the nutritional requirements of animals have been subjected to a tendency toward underestimation."

al. ('55) with respect to tryptophan-nicotinic acid. In this instance dietary tryptophan is constantly utilized for the biosynthesis of nicotinic acid-containing coenzymes, regardless of the presence or absence of dietary nicotinic acid.

From the manner in which muscle creatine content increases with age (fig. 1), it appears that a greater proportion of dietary arginine is diverted for creatine formation during the first 4 weeks of life than during the next 4-week period. Furthermore, a greater proportion of arginine is also available for growth than for maintenance purposes during the first few weeks of life. Both considerations go a long way in explaining the different arginine requirement of the chick before and after 4 weeks of age.

SUMMARY AND CONCLUSION

1. During the first 4 weeks of life, creatine biosynthesis is an important factor in the arginine metabolism of the chick.
2. The arginine requirement for growth and efficiency during the first three weeks of life is greater than 1.3%, possibly as high as 1.9%.
3. Before maximum values are reached, both growth and feed efficiency exhibit a unique plateau to increasing arginine supplementation.
4. The chick continues to divert arginine for creatine synthesis despite high muscle creatine levels produced by dietary creatine; consequently, there is little sparing of arginine by supplemental creatine.

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A COMPARISON OF THE BIOLOGICAL VALUES OF DIETARY PROTEIN INCORPORATED IN HIGH- AND LOW-FAT DIETS

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The thesis has been generally accepted that the chief function of carbohydrate or fat in the animal organism is calorigenic and that dietary carbohydrate is better than dietary fat as a protein-sparing nutrient. Even excessive amounts of fatty acids are oxidized through the tricarboxylic cycle in the presence of oxalacetic acid and in this way fatty acids of the dietary fat would become available for energy purposes. However, if a diet consists solely of fat, large amounts of ketone bodies would be produced and the kidney would probably produce more ammonia in order to neutralize these acids. The result may be an increased catabolism of the tissue protein. Literature on protein utilization provides conflicting evidence regarding the effect of dietary fat on protein metabolism. The discrepancies among the results of various laboratories seem, in part, due to ineffective experimental technics or to dubious interpretation of the data. Many research workers have used growth of the young animal or repletion of the protein-depleted animal to measure the nutritive value of a protein. The nitrogen balance index method of Allison and Anderson ('45) or the biological value method of Mitchell ('24) are among the more precise methods for measuring even small differences in protein quality.

[1] Government of Hyderabad (India) scholar.

The present investigation was undertaken to measure the effect of dietary fat on the utilization of protein for the protein-depleted rat and for the growing rat under conditions of isocaloric feeding, using Mitchell's ('24) method. The protein studied was casein.

MATERIALS AND METHOD

The composition of the experimental diets is shown in table 1. The low-fat (5%) and the high-fat (33%) diets were formulated in such a way that the ratio of protein calories (gross energy or heat of combustion) to non-protein calories was the same and that 1 gm of the low-fat diet and 0.75 gm of the high-fat diet contained equal amounts of protein, minerals, vitamins, crude fiber and gross energy. The low- and high-fat standardizing (low-nitrogen) diets compared closely with the low- and high-fat test diets, respectively, except for the protein content.

The protein used was vitamin-free casein.[2] The biological values of casein were measured for the protein-depleted rat and the growing rat using the Thomas-Mitchell procedure (Mitchell, '24). In both of the experiments the rats were fed isocalorically with respect to gross energy of the diets. In the first experiment protein-depleted rats were used. Thirty male albino rats, 120-day-old littermates, were offered Cannon's diet (3E), a low-nitrogen ration (Wissler et al., '46), individually at the rate of 20 gm per day for 84 days. Fourteen rats were paired according to their initial body weights and the loss of body weights during protein depletion. Three experimental periods were followed. Each period consisted of 17 days, of which the first 10 days were allowed for physiological adjustment to the test diets, and during the following 7 days excreta were collected. During the first experimental period pair mates were fed the test rations I and II (low or high in fat, respectively), and during the second experimental period they were fed the standardizing (low-

[2] Labco.

nitrogen) diets (III and IV, also low or high in fat). During the third experimental period reversal feeding of the test diets was followed so that the group which had received the low-fat diet in the first period now received the high-fat diet, and vice versa. The adequacy of a 10-day pre-feeding period

TABLE 1

Composition of the experimental diets

CONSTITUENTS	TEST DIETS		STANDARDIZING DIETS		TEST DIETS	
	Diet I	Diet II	Diet III	Diet IV	Diet V	Diet VI
	%	%	%	%	%	%
Whole egg [1]	...	...	5.00	6.67	...	...
Casein [2]	12.00	16.00	...	...	10.00	13.33
Salts 446 [3]	4.00	5.33	4.00	5.33	4.00	5.33
Sodium chloride	1.00	1.33	1.00	1.33	1.00	1.33
Vitaminized starch (691) [4]	5.00	6.67	5.00	6.67	5.00	6.67
Liver fraction (Wilson 1:20)	0.25	0.33	0.25	0.33	0.25	0.33
Cod-liver oil	1.50	2.00	1.50	2.00	1.50	2.00
Wheat germ oil	0.50	0.67	0.50	0.67	0.50	0.67
Wood flock [5]	2.00	2.67	2.00	2.67	2.00	2.67
Carbohydrate mixture [6]	70.75	34.33	77.82	44.39	72.75	37.00
Lard	3.00	30.67	2.93	29.94	3.00	30.67
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00
Analysis of diets after mixing (grams per 100 gm)						
Total nitrogen	1.75	2.33	0.61	0.82	1.47	1.93
Ether extract	4.89	32.71	5.20	33.57	5.12	33.41
Dry matter	92.87	95.81	93.39	94.81	92.68	95.76
Gross energy (calories)	401	558	391	545	398	553
Isocaloric factor	1.00	0.75	1.00	0.75	1.00	0.75

[1] Dried and ether-extracted.

[2] Labco, vitamin-free.

[3] Spector ('48).

[4] The percentage composition of vitaminized starch is as follows: calcium pantothenate 40 mg; pyridoxine hydrochloride, riboflavin and thiamine hydrochloride 5 mg each; nicotinic acid 20 mg; *p*-aminobenzoic acid 100 mg; inositol 200 mg; choline chloride (Merck dry mix) 8 gm; and cornstarch 91.625 gm.

[5] Distributed by Brown Co., Portland, Maine.

[6] Carbohydrate mixture consisted of cornstarch, cerelose and sucrose in the proportions of 3:3:1.

for physiological adjustment by the protein-depleted rat was established by measuring the daily urinary nitrogen excretion which plateaued off on the 7th day of feeding the test diets.

In the second experiment 14 male rats, 29-day-old littermates, were pair-fed test diets V and VI, low or high in fat, respectively. The plan of the experiment was similar to the one already described except that no third period of reversal feeding of pair mates on the test diets was used.

RESULTS AND DISCUSSION

Apparent digestibility. The nitrogen digestibility data obtained with the protein-depleted rats and with the growing rats are shown in table 2. Our data on casein digestibility do not support the observation of Geiger ('51) that simultaneously fed fat generally delayed the passage of food through the digestive tract and thus improved the digestion and utilization of the dietary protein. Shehata ('53) did not find any improvement in the digestibility of egg protein when the dietary fat was increased from 5 to 20%. Our data demonstrate a lack of influence of high dietary fat on the digestibility of casein for the protein-depleted rat as well as for the growing rat.

True digestibility. The determination of the true digestibility makes due allowance for the fecal nitrogen excreted on a nitrogen-free diet. The practice of reporting metabolic fecal nitrogen values per unit of dry matter consumed seems justified when the dry matter intakes of comparable diets do not differ appreciably. Mitchell ('34) found no increase in the metabolic fecal nitrogen when starch in the diet was largely replaced by an equal amount of fat. In the present investigation the dry matter intakes of the low-fat and the high-fat diets differed considerably because of isocaloric feeding. The metabolic fecal nitrogen values obtained per gram of dry matter consumed averaged 1.62 and 2.11 mg, respectively, for the low-fat and the high-fat diets for the

TABLE 2

Effect of fat level on the digestibility and utilization of dietary nitrogen

(average daily data of 7 rats with standard errors)

DIET	NITROGEN INTAKE	DRY MATTER INTAKE	APPARENT DIGESTIBILITY OF CASEIN	TRUE DIGESTIBILITY OF CASEIN	NITROGEN BALANCE	BIOLOGICAL VALUE
	mg	*gm*	%	%	*mg*	%
			Experiment on severely protein-depleted rats			
			Period I			
I (low-fat	210	11.51	89.2 ± 0.02	98.2 ± 0.51	89.3 ± 1.63	67.2 ± 0.33
II (high-fat)	210	8.33	89.2 ± 1.56	97.2 ± 0.85	88.2 ± 2.81	68.0 ± 0.43
Mean difference		3.18		1.0	1.1	0.8
P				$0.3 < P < 0.4$	$0.7 < P < 0.8$	$0.6 < P < 0.7$
			Period III			
II (high-fat)	209	8.33	90.4 ± 0.07	98.7 ± 0.99	76.8 ± 1.3	64.4 ± 0.91
I (low-fat)	209	11.51	89.4 ± 0.46	98.4 ± 1.07	85.9 ± 3.1	69.5 ± 1.75
Mean difference			1.0	0.3	9.1	5.1
P			$0.05 < P < 0.1$	$P = 0.8$	$0.01 < P < 0.02$	$0.02 < P < 0.05$
			Experiment on growing rats			
VI (low-fat)	125	7.88	90.2 ± 0.27	99.5 ± 0.27	57.5 ± 1.1	72.4 ± 1.2
VII (high-fat)	123	6.11	90.9 ± 0.28	99.6 ± 0.18	55.0 ± 0.9	69.2 ± 0.8
Mean difference	2	1.77	0.7	0.1	2.5	3.2
P			$0.05 < P < 0.1$	$0.6 < P < 0.7$	$0.1 < P < 0.2$	$0.05 < P < 0.1$

protein-depleted rats and 1.57 and 1.77 mg, respectively, for the growing rats. In either case the metabolic fecal nitrogen per gram dry matter consumed was significantly greater for the high-fat group, particularly in the protein-depleted rat. Whether the significant increase in the metabolic fecal nitrogen per gram of dry food intake is due to the high fat content of the diet or to a concomitant reduction in food intake was investigated in a separate experiment. When given equal amounts of food, metabolic fecal nitrogen values per gram of food intake for growing rats were found to be 1.56 ± 0.086 (s.e.) on the low-fat diet and 1.56 ± 0.038 on the high-fat diet. The data demonstrate that the excretion of the metabolic nitrogen in the feces is directly related to the dry matter intake and not to the fat percentage of the diet.

In view of these data the authors found it justifiable to calculate the true digestibility and biological values of casein for protein-depleted rats on the low-fat and the high-fat test diets in the third experimental period, using the mean values of metabolic fecal nitrogen per gram of low- and high-fat standardizing diet, respectively. No significant difference in the true digestibility of casein was observed on the low-fat and the high-fat diets for the growing rat as well as for the depleted rat.

Nitrogen balance. By definition "nitrogen balance" is the difference between nitrogen intake and nitrogen excreted in urine and feces. The average daily nitrogen balance figures obtained in both the experiments on the depleted rats and the growing rats indicate no beneficial effect of the high-fat diet on protein utilization (see table 2). In fact, the nitrogen balances are significantly in favor of the high-carbohydrate diet in the third period of collection on the depleted rats ($P < 0.02$).

Endogenous urinary nitrogen. No beneficial effect of dietary fat, compared to carbohydrate, on endogenous protein metabolism was observed in the protein-depleted rat or in the growing rat. These results agree with the data of McCollum and Hoagland ('13) who fed pigs using fat as the sole source

of energy and found no sustained rise in the urinary nitrogen output of animals which had been reduced to their lowest possible level of nitrogen metabolism by long-continued starch feeding. But Zagami's ('30) experiments on two pigs weighing 16 kg reveal a significant rise in urinary nitrogen on a nitrogen-free diet if carbohydrate in the diet is largely replaced by fat. This investigator, however, collected urine immediately after shifting the animals from a low-fat diet to a high-fat diet and thus did not allow for physiological adjustment to the test diet. The importance of using the adjustment period before the excreta are collected for nitrogen determination has been recently stressed by Thomson and Munro ('55), who observed an immediate rise in the urinary nitrogen following the replacement of the dietary carbohydrate by fat. The rise in the urinary nitrogen was found to persist for 4 to 5 days, after which period the excretion of nitrogen returned to a significantly lower and sustained level. Thus Zagami's conclusion that increasing dietary fat would increase tissue catabolism is the unfortunate result of faulty experimental procedure.

The endogenous urinary nitrogen per unit of body weight raised to the three-fourths power when the diets were fed on an isocaloric basis was 0.58 and 0.61 mg, respectively, for the low-fat and the high-fat groups of growing rats, and 0.66 and 0.59 mg, respectively on the low-fat and the high fat diets for the depleted rats (see table 3). The differences were not significant in either experiment.

Fox et al. ('52) have observed that the rat receiving fat in the diet does a seemingly better job in adjusting to the stress of caloric restriction in protein starvation than its carbohydrate-fed control. These research workers have attributed an extra-caloric function to dietary fat (Swanson, '51). Our rats were offered experimental diets to provide caloric needs for maintenance and growth (or repletion), assuming the net energy of the diets to be 96% (Hamilton, '39) of the metabolizable energy and the metabolizable energy to be 90% of the gross energy (Metta and Mitchell, '54) of the

TABLE 3

Effect of isocaloric feeding of low-nitrogen diets, high or low in fat, on the excretion of nitrogen in the urine and feces

(average daily data of 7 rats with standard errors)

DIET	AVERAGE BODY WT.	DRY MATTER INTAKE	METABOLIC FECAL N	METABOLIC FECAL N PER GM DRY FOOD	ENDOGENOUS URINARY N	EDOGENOUS URINARY N PER GRAM WT $^{3/4}$
	gm	*gm*	*mg*	*mg*	*mg*	*mg*
			Experiment on severely protein-depleted rats			
III (low-fat)	219	11.50	18.6 ± 0.74	1.62 ± 0.064	33.2 ± 1.6	0.58 ± 0.02
IV (high-fat)	233	8.32	17.4 ± 0.44	2.11 ± 0.049	36.2 ± 1.4	0.61 ± 0.03
Mean difference		3.18	1.2	0.49	3.0	0.029
P			$0.1 < P < 0.2$	$P < 0.001$	$0.1 < P < 0.2$	$0.3 < P < 0.4$
			Experiment on growing rats			
III (low-fat)	114	9.23	14.5 ± 0.74	1.57 ± 0.080	23.5 ± 1.5	0.66 ± 0.05
IV (high-fat)	120	7.21	12.8 ± 0.40	1.77 ± 0.056	22.1 ± 1.1	0.59 ± 0.03
Mean difference	6	2.02	1.7	0.20	1.4	0.063
P			$0.05 < P < 0.1$	$0.05 < P < 0.1$	$0.4 < P < 0.5$	$0.2 < P < 0.3$

diets. The daily caloric needs for maintenance (Q) were estimated from Brody's generalized equation, $Q = 70.4\ W_{kg}^{.734}$ for basal energy (Brody and Proctor, '32), to which was added 25% for activity increment (Mitchell and Carman, '26). Energy requirements for growth were provided for by including an additional 12 dietary calories per rat per day, assuming a daily body weight gain of 4 gm. No beneficial effect of fat as compared with carbohydrate was observed under our experimental conditions of satisfactory and equal caloric (gross energy) intake.

Biological values of casein. The high-carbohydrate diet was observed to be as good as the high-fat diet for the protein-depleted rat as far as the retention of the truly absorbed nitrogen was concerned. The biological values of casein for the low-fat and the high-fat groups of rats were 67.2 and 68.0%, respectively, during the first collection period, and 69.5 and 64.4%, respectively, during the third collection period (see table 2). In the third period the differences were statistically significant (P between 0.02 and 0.05) in favor of the low-fat diet. Similarly, the biological values of casein for growing rats were not significantly improved due to isocaloric replacement of most of carbohydrate by fat. The biological values were found to be 72.4% for the low-fat group and 69.2% for the high-fat group.

Forbes and his associates had carried out a series of experiments on rats to investigate the effect of dietary fat on economy of food utilization. In their first series of experiments ('46a, b) they used 2, 5, 10, and 30% fat in the test diets which contained 22% protein. They fed growing rats isocaloric, isonitrogenous amounts of food and concluded from their data that "these observations signify that increasing fat content of the isocaloric diets favor both the digestion and the fundamental utilization of the protein present." It is true that the apparent digestibility of protein was found to increase in the increasing order of the fat contents of the diets. The validity of the conclusions is, however, questionable, since they used a high protein content (22%) in their test

diets, which also had narrow nutritive ratio (about 1:3.5). In a second series of experiments ('46c, d) these investigators used the same diets, in which a supply of certain vitamins was greatly augmented, and found no favorable effect of dietary fat on nitrogen retention. Contrary to their previous findings the carcass nitrogen of the rats decreased with increasing fat in food. The authors themselves go on to say, "It is unnecessary to attempt a fundamental interpretation of this difference in evidence relating to nitrogen utilization, first because it was not technically positive, and second because the relative proportions of protein, carbohydrate and fat catabolized may vary considerably . . . ." In a third series of experiments (French et al., '48) the nitrogen retention was not found to be influenced when fat was exchanged for some of the dietary carbohydrate at low levels of protein (7%) intake. From the conflicting data of these investigators, it is thus obvious that carbohydrate and fat when fed isocalorically are essentially similar in function with respect to protein utilization.

Pearson and Panzer ('49) equalized protein and energy intake in growing rats fed fat-free and fat-containing diets and observed no significant difference in growth. Though significantly less valine and methionine were excreted in the urine of rats on fat-containing diets (the difference in the amino acids excreted being 0.27 and 0.08 mg per rat per day, respectively) the differences are very small and therefore should not be interpreted to mean that the fat "in the diet of the rats enhanced the efficiency with which valine and methionine are utilized."

Working with very young rats Shehata ('53) observed a slight (3%), but significantly better, utilization of dietary protein on a low-fat diet. But during the later phase of growth of the rats he did not find any difference in the biological value of egg protein when dietary carbohydrate was isocalorically replaced with fat. Thus Shehata's data (except for data in one experiment) may also be interpreted to mean that fat,

on the isocaloric basis, is as good as carbohydrate with respect to the digestion and retention of dietary protein.

SUMMARY AND CONCLUSIONS

The effect of fat as compared to isocaloric amounts of carbohydrate on nitrogen utilization in the severely protein-depleted and the growing rat was investigated using the Thomas-Mitchell procedure. The low-fat and the high-fat diets contained, respectively, 5 and 33% fat, mainly lard. Casein was the dietary protein tested. The rats were fed the test diets so that they received equal amounts of protein, crude fiber, vitamins, minerals and gross energy, the main difference between the two groups being in the fat intake.

When fed in isocaloric amounts, dietary fat and carbohydrate have essentially the same effect on protein utilization in both the protein-depleted rat and the growing rat.

Neither the apparent digestibility nor the true digestibility of casein is improved by the higher dietary fat level.

Fat does not seem to spare endogenous nitrogen under conditions of satisfactory and equal caloric intake.

Higher dietary fat is not found to improve the biological value of casein. In fact, the indications in one experiment are that a high-carbohydrate diet tends to improve the biological value of casein in the protein-depleted rat.

The metabolic fecal nitrogen per gram food intake increases significantly when the dietary fat is increased because of a concomitant reduction in the food intake due to isocaloric feeding. This value, however, remains constant irrespective of the fat content of the diet if the rats are fed equal amounts of the low- and the high-fat diets.

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A STUDY OF BLOOD AND URINARY VITAMIN CONCENTRATIONS AND OF FAT BALANCE IN THE RHESUS MONKEY (*MACACA MULATTA*) [1]

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Despite the usefulness of the monkey as an experimental animal, there are relatively few studies of the biochemistry and physiology of this species. In 1944 Day ('44) commented on the lack of investigations of the nutritional requirements of the monkey. According to Ruch ('41), until 1941 only 45 reports had been published concerning the nutrition of this animal.

Waisman and Elvehjem ('43) and Waisman et al. ('43) maintained the rhesus monkey on a purified diet and omitted individual vitamins in order to study possible deficiencies. Hematologic observations were made. Replacement of the missing vitamin alone was insufficient to restore normal blood values, but restoration followed the addition of milk or a liver fraction.

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Mann et al. ('52) kept cebus monkeys on a purified diet and recorded a series of normal values for various constituents of blood in this species. Riboflavin was the only vitamin for which normal values were reported in this study.

During the last three years experiments on vitamin deficiencies and on the metabolic effects of irradiation in the monkey *(Macaca mulatta)* have been under way in our laboratory. The animals were kept on a purified diet and studies were made of the blood levels and urinary excretion of several vitamins. Because of an interest in lipid absorption, fat balances have been determined. This paper reports values obtained in these determinations and records additional average hematologic values observed in normal monkeys during the experimental period.

EXPERIMENTAL

Upon their arrival from dealers the monkeys were kept in isolation for a period of about 4 weeks. Healthy monkeys weighing between 3.0 and 4.5 kg were then transferred to the regular quarters and kept in individual cages. Preceding the experimental period they were fed a stock laboratory mixture [3] supplemented with 50 mg of ascorbic acid daily. After a suitable length of time (usually about one month) the animals were offered the purified diet which had been mixed with some thin slices of banana. This was well accepted by the end of one or two weeks, after which the amount of banana was gradually but progressively reduced and finally omitted from the diet. As soon as the animals accepted the purified diet, they were trained to consume their ration within a two-hour period. Thus, food was made available only once a day for a two-hour period, but water was allowed ad libitum. A 50 mg tablet of ascorbic acid was given to each animal at mealtime. A period of 4 to 6 weeks was allowed for adjustment to the diet and to the feeding schedule before any analytical measurements

[3] Okatie Farms Monkey Diet.

were performed. The composition of the diet [4] is given in table 1. It is similar to that used by Waisman and Elvehjem ('43). The dry components were mixed every 5 days and stored in stoppered bottles. Vitamins A, D and E were added to cottonseed oil, and this mixture was kept in the refrigerator. The oil and dry ingredients were mixed daily for feeding.

TABLE 1

Composition of purified diet

INGREDIENT	AMOUNT
	gm
Casein, vitamin-free [1]	900
Salt mixture [2]	200
Sodium chloride	50
Sucrose	3,450
Vitamin mixture [3]	47.5
Choline chloride mixture [4]	27.5
Pteroylglutamic acid mixture [5]	50
Vitamin B_{12} mixture [6]	25
Oil mixture [7]	250
Ascorbic acid (added to each daily feeding as a 50 mg tablet)	

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.

[2] Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., J. Nutrition, *14:* 273, 1937.

[3] Composition in grams: riboflavin 1.0; thiamine·HCl 1.0; pyridoxine·HCl 1.0; calcium pantothenate 10.0; niacinamide 25.0; biotin 0.02; inositol 100.0; 2-methyl-1,4-naphthoquinone 0.50; sucrose 811.5.

[4] Composition in grams: choline chloride 100; sucrose 1000.

[5] Composition in grams: pteroylglutamic acid 0.02; sucrose 100.

[6] Composition in grams: vitamin B_{12} 0.0002; sucrose 160.

[7] Composition in grams: Squibb's Navitol with Viosterol 1.0 (65,000 I.U. vitamin A and 13,000 I.U. vitamin D); Natopherol acetate 1.0 (146 mg d-α-tocopherol acetate); cottonseed oil 98.0.

To obtain a satisfactory consistency, water (approximately 15% of the diet weight) was added and the diet was mixed in an electric food mixer. Daily intake was estimated by weighing the food offered the monkeys and that remaining at the end of the feeding period. Adequacy of this diet for

[4] We are indebted to Lederle Laboratories, Inc., Pearl River, New York; to Merck and Company, Inc., Rahway, New Jersey; to Hoffmann-La Roche, Inc., Nutley, New Jersey; and to Distillation Products Industries, Rochester, New York, for generous supplies of vitamins used in these studies.

normal growth and maintenance of the species had been established by feeding animals on the diet for periods up to three years. These monkeys have exhibited a good increase in weight and have maintained healthy coats and hematologic levels.

Blood values for vitamin A, carotene, and ascorbic acid were determined by the micro procedures described by Bessey et al. ('46) and by Lowry et al. ('45). Thiamine, riboflavin, N^1-methylnicotinamide, and ascorbic acid were measured in urine by chemical methods reported by Perlzweig et al. ('45), Ferrebee ('40), Huff, Perlzweig and Tilden ('45), and Roe and Kuether ('43), respectively. The microbiological procedure of Atkin et al. ('43) was used to determine the vitamin B_6 group in urine. Urinary pteroylglutamic acid determinations were done according to the microbiological method described by the Association of Official Agricultural Chemists ('50) using *Streptococcus fecalis.* The procedure employed for urine collection was as follows: at the end of the two-hour feeding period the bottom tray of the cage was washed in order to remove spilled food. Collection into bottles containing either a 20% solution of metaphosphoric acid or acetate buffer, pH 4.8, was then started and continued for 24 hours. Each 24-hour specimen from one monkey constituted a single sample.

Fecal fatty acids were determined by the method of van de Kamer et al. ('49) with minor modifications. Each sample of feces, constituting a three-day collection period for a single monkey, was homogenized with 95% ethyl alcohol in a Waring blendor, diluted to a definite volume, and appropriate aliquots were used for fecal fatty acid analysis and for dry weight determinations.

RESULTS AND DISCUSSION

Table 2 summarizes the average intake and excretion of thiamine, riboflavin, N′-methylnicotinamide, pteroylglutamic acid, vitamin B_6, and ascorbic acid for 14 monkeys in three

separate experimental groups during a period of 5 to 7 days. It is noted that for the same intake of the vitamins, a greater quantity of riboflavin was excreted than of thiamine. This is in keeping with experience in the human. Only about 1 mg of N^1-methylnicotinamide was excreted daily, although the average intake of niacinamide was relatively high (20 to 30 mg/day). In humans, Sarett, Huff and Perlzweig ('42)

TABLE 2

Average intake and excretion of vitamins by three groups of healthy monkeys

VITAMIN	NUMBER OF ANIMALS	INTAKE		URINARY EXCRETION	
		Average ± S.D.[1]	No. of values[2]	Average ± S.D.[1]	No. of values[2]
		mg/day		*mg/day*	
	4	1.01 ± 0.31	28	0.185 ± 0.62	28
Thiamine	4	0.96 ± 0.27	20	0.183 ± 0.66	19
	6	0.78 ± 0.23	44	0.134 ± 0.59	31
	4	1.01 ± 0.31	28	0.362 ± 0.078	28
Riboflavin	4	0.96 ± 0.27	20	0.385 ± 0.122	19
	6	0.78 ± 0.23	44	0.213 ± 0.072	32
Ingested as					
niacinamide	4	25.3 ± 7.7	28	1.31 ± 0.49	28
(Excreted as	4	24.1 ± 6.8	20	1.34 ± 0.69	19
N'-methylnico-	6	19.4 ± 5.8	44	0.78 ± 0.61	30
tinamide)					
Pteroyl-	4	0.204 ± 0.066	28	0.011 ± 0.005	19
glutamic acid	4	0.198 ± 0.056	20	0.014 ± 0.010	18
	6	0.160 ± 0.047	38	0.003 ± 0.003	32
	4	1.01 ± 0.31	28	0.0254 ± 0.0139	18
Pyridoxine	4	0.96 ± 0.27	20	0.0376 ± 0.0180	18
	6	0.78 ± 0.23	44	0.0126 ± 0.0054	31
Ascorbic	4	50 ± ...	28	2.68 ± 1.10	28
acid[3]	4	50 ± ...	19	2.44 ± 0.79	19
	6	50 ± ...	31	2.58 ± 0.87	31

[1] S.D. = standard deviation = $\sqrt{\dfrac{\Sigma x^2 - \dfrac{(\Sigma x)^2}{n}}{n-1}}$.

[2] Number of values from which average and standard deviation were calculated.

[3] Fed as a 50 mg tablet.

and Coulson, Ellinger and Smart ('45) found that N^1-methylnicotinamide represents about 20% of the excretory products of niacin metabolism.

It is interesting that only 2 to 3 mg of ascorbic acid were excreted on a daily intake of 50 mg. This suggests a rather high requirement of this species for this vitamin. In early studies Day et al. ('35) and Langston et al. ('38), using less-

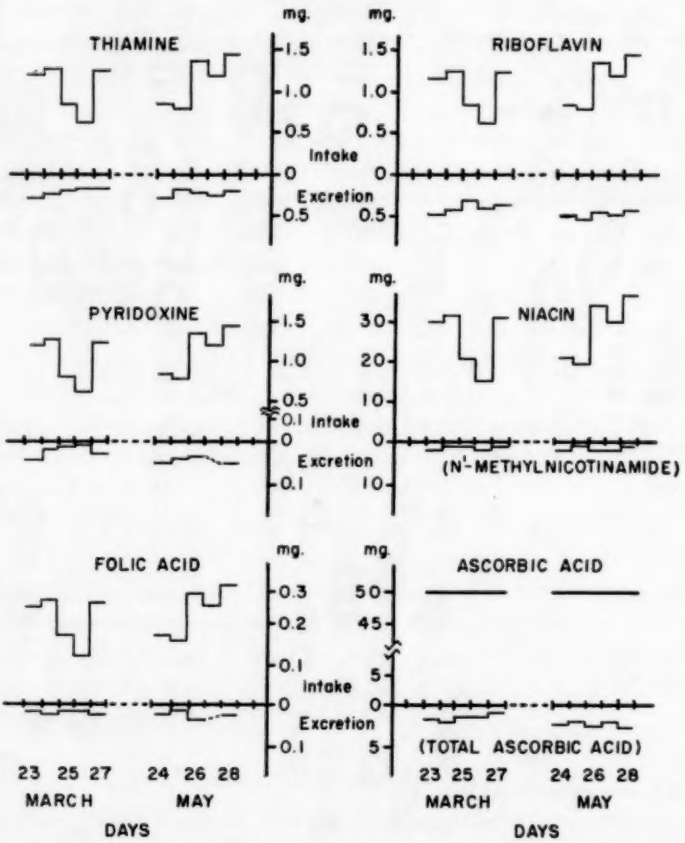

Fig. 1 Typical daily intake and 24-hour urinary excretion of vitamins by a monkey during two periods of 5 days.

purified diets, employed supplements of ascorbic acid in amounts up to about 10 mg. The possibility that these levels represent suboptimal intakes for this species is currently under study in this laboratory.

On an intake of about 0.2 mg/day the average excretion of pteroylglutamic acid ranged from 0.003 to 0.014 mg/day.

The daily variation encountered in these monkeys is illustrated by the daily intake and urinary excretion of 6 vitamins studied in one monkey during two 5-day periods (fig. 1).

The results of determinations of blood vitamin levels may be summarized as follows: averages of 197 I.U. % of vitamin A (S.D. = 52) and of 6 μg % of carotene (S.D. = 3) were found in 21 determinations made on 10 monkeys. Concentration of ascorbic acid in the serum averaged 0.77 mg % (S.D. = 0.10) in 42 determinations in 10 monkeys. These values for vitamins A and C were reasonably constant and are at levels similar to those found in the well-fed human. Such findings may be further evidence of the appropriateness of the monkey as an experimental counterpart to man for studies of deficiencies of these vitamins. The low carotene value is consistent with the absence of this factor from the diet.

The ability of the monkey to absorb fat from the intestine is indicated by the fat balances recorded in table 3. On a diet containing 5% of cottonseed oil the range in fecal fat loss (as percentage of ingested fat) was from 2.76 to 8.54 for 9 monkeys. One animal exhibited a much higher loss (17%) of the ingested amount despite an intake similar to that of the other animals. The excessive fat content of the feces in this monkey was also manifested as a high value in fecal fat concentration when calculated as percentage of dry weight of feces (19.2 compared to the range 6.13 to 13.4 for the other monkeys). No explanation of this variant was apparent.

Although there were differences in the intake of food (and thus of fat) in each monkey from day to day, the variation in fecal loss of fat was more pronounced than expected from studies on other species. In most of the monkeys the calcu-

TABLE 3

Fat intake and excretion in normal monkeys

MONKEY NUMBER	NUMBER OF COLLECTION PERIODS [1]	DAILY FATTY ACID INTAKE	FECAL FAT CONCENTRATION	FECAL FAT EXCRETION
		Average ± S.D.[2]	Average ± S.D.[2]	Average ± S.D.[2]
		gm	*% of dry weight of feces*	*% of ingested fat*
7	25	5.91 ± 1.09	7.95 ± 2.60	2.76 ± 1.30
8	25	4.57 ± 0.97	6.54 ± 2.12	3.18 ± 1.58
9	25	4.57 ± 0.47	8.15 ± 2.04	3.73 ± 1.39
10	25	3.93 ± 0.74	6.13 ± 2.39	3.54 ± 1.97
30	7	5.10 ± 0.68	13.00 ± 7.21	5.63 ± 4.78
31	7	3.36 ± 0.81	6.60 ± 3.40	4.02 ± 1.88
32	12	3.43 ± 0.87	13.40 ± 9.15	8.54 ± 5.60
33	12	4.90 ± 1.74	8.73 ± 3.02	4.25 ± 2.10
34	7	3.29 ± 0.91	7.81 ± 3.40	6.45 ± 3.21
35	7	3.45 ± 0.85	19.21 ± 5.85	17.06 ± 5.67

[1] One collection period = 3 consecutive days.

[2] S.D. = standard deviation = $\sqrt{\frac{\Sigma\chi^2 - \frac{(\Sigma\chi)^2}{n}}{n-1}}$.

TABLE 4

Fecal fat concentration and excretion for monkeys 32 and 35

COLLECTION NUMBER	MONKEY 32		MONKEY 35	
	Fecal fat concentration	Fecal fat excretion	Fecal fat concentration	Fecal fat excretion
	% of dry wt. of feces	*% of ingested fat*	*% of dry wt. of feces*	*% of ingested fat*
1	4.14	1.38	23.7	18.0
2	14.3	17.3	23.8	24.9
3	10.9	3.48	17.0	19.9
4	1.55	1.33	7.13	7.0
5	36.4	16.9	22.2	13.0
6	17.8	10.9	21.0	19.5
7	19.3	10.0	19.7	16.5
8	8.67	4.27	...	...
9	13.3	11.9	...	...
10	4.78	4.20	...	...
11	16.1	12.1	...	...
12	13.5	8.80	...	...

lated average and standard deviation describe the distribution of the variation. This is not true for monkeys 30 and 32 in which cases the average value was weighted greatly by extremes. The data for one of these are presented in detail (table 4). On the other hand, a more typical case is illustrated in figure 2, which shows fat excretion and fat intake for monkey 9. It may be seen that many values are grouped around the calculated average (3.73% of ingested fat). Simi-

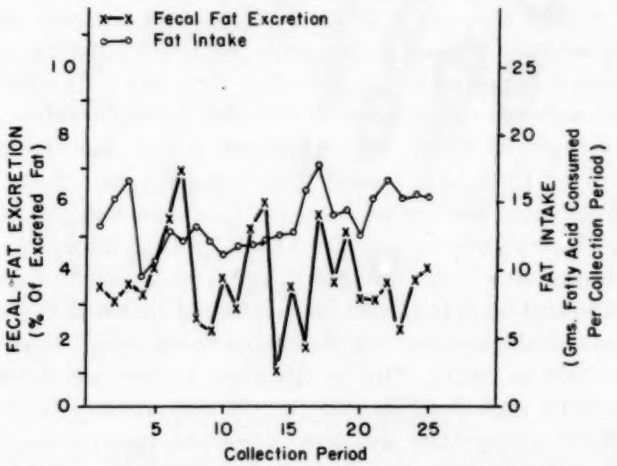

Fig. 2 Fat intake and fecal fat excretion of a monkey (no. 9) typical of the group studied.

lar data for monkey 35 are shown in table 4. This is the animal which excreted consistently larger amounts of fat than the others. Despite the unusually high excretion, the calculated average and standard deviation present a true picture of the distribution of the values of the individual collection periods.

Variation in fat excretion occurred in each monkey from one collection period to another, but with the exception of animal 35 the over-all averages do not differ greatly from one animal to another. A long balance period obviously pro-

vides a more reliable index of fat absorption than a balance taken over a short period of time (e.g., three days) — a principle well established for studies in the human.

Although it might be expected that the percentage excretion of fat would vary with intake, this did not occur within the range of intakes studied in these animals. The food intake may be calculated readily from the data on fatty acid intake listed in table 3 by multiplying by 20.

During the experimental periods the monkeys maintained their weight and, in some cases, there was a slight gain.

Hematologic studies were made at the beginning of the experimental period and at intervals thereafter. Insignificant variations were encountered during this time; therefore, only the averages of all the determinations (three determinations on each of 10 monkeys) and their standard deviations need be cited. These were as follows: erythrocyte count 5.28 million (S.D. = 0.77); leucocyte count 14,400 (S.D. = 5500); packed-cell volume 40.5% (S.D. = 2.20); and hemoglobin 12.2 gm/100 ml (S.D. = 1.08). It should be mentioned that monkey 35 exhibited an abnormally low leucocyte count at all times (i.e. about 3000 to 5000). This is the same animal which showed abnormally high fecal loss of fat. If this monkey is omitted from the group, the average leucocyte count was 15,600 (S.D. = 4500).

SUMMARY

A study of blood levels, of the urinary excretion of several vitamins, and of fat balance has been made in apparently healthy rhesus monkeys kept on a purified diet.

For the same daily intake of thiamine, riboflavin, and pyridoxine (about 1 mg/day) there was excreted a greater quantity of riboflavin (about 0.32 mg/day) than of pyridoxine (about 0.025 mg/day) or of thiamine (about 0.17 mg/day). On an intake of 20 to 30 mg/day of niacinamide about 1 mg/day was excreted as N^1-methylnicotinamide. On a daily ascorbic acid intake of 50 mg only 2 to 3 mg/day of ascorbic

acid were found in the urine. The average excretion of pteroylglutamic acid ranged from 0.003 to 0.014 mg/day on an intake of about 0.2 mg/day.

Determinations on serum samples revealed average levels of about 197 I.U. % of vitamin A, 6 μg % of carotene (carotene-free diet), and 0.77 mg % of ascorbic acid.

With 5% fat in the diet only 2.8 to 8.5% of the ingested fat was lost in the feces. The fecal fat concentration ranged from 6.1 to 13.4% of the dry weight of the feces. Because of the variation in fecal fat loss in an individual monkey from one collection period to another, a long balance period is a more reliable index of fat absorption than a short-time study.

On this diet body weight was maintained or increased and hematologic values were similar to those commonly reported for monkeys.

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THE EFFECTS OF X-IRRADIATION ON THE VITAMIN EXCRETION OF THE RHESUS MONKEY *(MACACA MULATTA)* [1]

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INTRODUCTION

There are scattered reports concerning the effect of x-irradiation upon various aspects of vitamin metabolism. Ascorbic acid, because of its relationship to endocrine function and to stress, has been studied most frequently. For example, Kretzschmar and Ellis ('47) reported that human patients usually exhibited lower serum ascorbic acid levels following x-irradiation therapy. This effect was also observed in rabbits, a species which does not require this vitamin. Monier and Weiss ('52) found a significant increase in the urinary excretion of dehydroascorbic acid and diketogulonic acid but not of the reduced form of vitamin C following the exposure of rats to 800 r of x-irradiation. Venters and Painter

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('51) found an increase in the pituitary and a decrease in the adrenal ascorbic acid levels of x-irradiated rats. The latter change is greater in male than in female rats (Wexler, Pencharz and Thomas, '52) and, in general, irradiation-induced tissue changes vary with the strain of rat (Oster, Kretchmar and Bethell, '53).

Dunlap and Robbins ('43) found no differences in the thiamine content of blood or liver of the mouse that could be attributed to irradiation. Irradiation of the pituitary in man does not seem to alter urinary excretion of thiamine (Marzocchi and Barbieri, '51).

Jarvis and Cayer ('49), working with patients with malignant diseases, were unable to detect any increase in the riboflavin and N^1-methylnicotinamide excretion after x-ray treatment. They noted, however, that the patients who suffered the most ill effects from x-ray therapy had significantly lower levels of excretion of these two substances.

A study of the excretion of several B vitamins in rats exposed to x-irradiation has been reported by Pearson et al. ('53), who have demonstrated an increase in the urinary excretion of pteroylglutamic acid and of niacin as a primary irradiation effect. A less striking increase in pyridoxine excretion has been shown to be related to food restriction (Song, Pearson and Darby, '56).

The nutritional requirements of the monkey make it appear to be a valuable subject for the study of irradiation damage. There is, however, but limited information on this animal. This report deals with the blood ascorbic acid levels and the excretion of riboflavin, thiamine, N^1-methylnicotinamide, pteroylglutamic acid, pyridoxine, and ascorbic acid following exposure of this species to x-irradiation.

EXPERIMENTAL

Ten adult female monkeys *(Macaca mulatta)* of approximately 4 kg mean weight were caged, fed a complete purified diet, and maintained as described in the accompanying report

(Dutra de Oliveira et al., '56). The animals were kept on this regimen for about three months before irradiation and appeared to be healthy during this period.

For exposure to x-irradiation the animals were placed singly in a masonite box. Air doses of x-irradiation of 325, 400 or 650 r, depending on the experiment, were administered. The 650 r dose was delivered in two parts of 325 r each with a 5-minute interval between doses. The Keleket 220 Kv. x-ray machine was calibrated before use, and when operated at 20 ma with ½ mm Cu and 1 mm Al filtration, delivered 16 r/min. at a 78 cm target distance. The control animals were always placed in the same box in order more nearly to equate the handling of both groups.

After irradiation the animals were pair-fed as follows: food was made available to the irradiated group for a two-hour feeding period, the amount consumed measured, and a similar portion given to the controls. The animals had previously been trained to consume their total daily intake during a two-hour period.

In the first experiment two animals were given 325 r of irradiation and two were used as pair-fed controls. A few months later, the non-irradiated controls were exposed to 400 r and those animals previously irradiated (now recovered) were retained as pair-fed controls. The second experiment was performed on a separate group of 6 monkeys. Of these, three were irradiated at the 650 r level and three were kept as pair-fed controls.

All of the vitamin determinations were made by the chemical and microbiological methods indicated in a previous report (Dutra de Oliveira et al., '56) except for ascorbic acid. Initially the procedure of Roe et al. ('48) was used to determine reduced ascorbic acid, dehydroascorbic acid, and diketogulonic acid. In later experiments only total ascorbic acid was determined because of the questionable specificity of the method for the estimation of diketogulonic acid (Misra, '53).

RESULTS

The response of the animals to the doses of 325 r and 400 r was similar. Following exposure they became inactive but diarrhea was not usually observed. Anorexia of one to three days duration was followed by gradual return of appetite until pre-irradiation levels of consumption were reached within one to two weeks. The mean daily intake during the first three days after irradiation was 52 gm as compared with 106 gm prior to irradiation. No tendency to hemorrhage was observed in this group. Some hair loss occurred.

The higher dose of irradiation (650 r) produced similar but more severe initial effects. Anorexia was more prolonged and severe during the first week after irradiation, in which the mean daily intake was reduced from 95 gm prior to irradiation to 25 gm. A month was required for the two surviving animals to return to their initial levels of dietary intake. One monkey developed a persistent epistaxis on the 11th post-irradiation day. Because death of the monkey was imminent, it was sacrificed 13 days after irradiation. The principal gross findings at autopsy were intestinal obstruction, probably subsequent to fluid transudation with organization of the fibrin, and lung hemorrhages.

The leucopenia occurring in monkeys after irradiation is described elsewhere (Coniglio et al., '56). The decline in leucocyte levels was especially severe in the 650 r group.

The urinary excretion of thiamine, riboflavin, and N^1-methylnicotinamide showed considerable daily variation and a decrease after irradiation at all levels of treatment. The condensed data are presented in table 1. The pattern of excretion of these three vitamins was similar, and where decreased excretion followed irradiation, parallel decreases resulted from the dietary restriction imposed upon the pair-fed controls.

Daily urinary loss of diketogulonic acid as estimated by the Roe procedure showed a rise in excess of 2 mg (table 2) in all of the irradiated monkeys on the first day after irradiation. This level was significantly above that of the pre-

TABLE 1

Mean daily urinary excretion of thiamine, riboflavin and N¹-methylnicotinamide for 4 monkeys irradiated at 325–400 r and three monkeys irradiated at 650 r and their controls

VITAMIN		IRRADIATED AT 325 TO 400 r GROUP MEAN EXCRETIONS IN MG/DAY			IRRADIATED AT 650 r GROUP MEAN EXCRETIONS IN MG/DAY		
		6 days prior to irradiation	First day after irradiation	5 days after irradiation [1]	4 days prior to irradiation	First day after irradiation	5 days after irradiation [1]
Thiamine	I [2]	0.17 (24) [3]	0.22 (4)	0.11 (20)	0.12 (16)	0.11 (3)	0.05 (15)
	PFC [2]	0.19 (23)	0.12 (4)	0.13 (20)	0.14 (16)	0.11 (3)	0.07 (15)
Riboflavin	I	0.35 (24)	0.36 (4)	0.23 (20)	0.23 (16)	0.26 (3)	0.13 (15)
	PFC	0.40 (23)	0.24 (4)	0.26 (20)	0.20 (16)	0.18 (3)	0.13 (15)
N¹-methyl-nicotinamide	I	1.28 (24)	0.93 (4)	0.93 (20)	0.79 (14)	0.55 (3)	0.37 (14)
	PFC	1.37 (23)	1.18 (4)	1.10 (20)	0.81 (16)	0.49 (3)	0.42 (15)

[1] The first day after irradiation included.
[2] I = irradiated group. PFC = pair-fed control group.
[3] The figures in parentheses indicate the number of individual determinations from which each mean was calculated.

TABLE 2

Mean daily urinary excretion of diketogulonic acid

ANIMAL NO.		IRRADIATED AT 325 r MEAN DAILY EXCRETIONS IN MG/DAY			ANIMAL NO.		IRRADIATED AT 400 r MEAN DAILY EXCRETIONS IN MG/DAY		
		7 days prior to irradiation	First day after irradiation	6 days after irradiation [1]			5 days prior to irradiation	First day after irradiation	6 days after irradiation [1]
7	PFC [2]	0.72 (7) [3]	1.01	1.00 (6)	8	(PFC)	1.02 (5)	0.46	0.67 (4)
10	PFC	0.88 (7)	0.46	0.84 (6)	9	(PFC)	0.61 (4)	0.87	0.51 (4)
8	I [2]	0.76 (7)	2.94	0.85 (6)	7	(I)	1.19 (5)	6.29	0.90 (4)
9	I	0.54 (7)	2.34	0.78 (6)	10	(I)	0.96 (5)	2.19	0.58 (4)

[1] The first day after irradiation included.
[2] I = irradiated monkey. PFC = pair-fed control monkey.
[3] The figures in parentheses indicate the number of daily determinations from which each mean was calculated.

TABLE 3

Mean daily urinary excretion of "total" ascorbic acid and serum ascorbic acid concentration in irradiated monkeys

IRRADIATION DOSE	ANIMAL NO.	DAYS PRIOR TO IRRADIATION		FIRST DAY AFTER IRRADIATION		ALL DAYS AFTER IRRADIATION [1]	
		Urinary excretion	Serum level	Urinary excretion	Serum level	Urinary excretion	Serum level
		mg/day	*mg %*	*mg/day*	*mg %*	*mg/day*	*mg %*
325 r	7 PFC [2]	3.00 (7) [3]	0.78 (2)	3.55	0.86	4.63 (6)	0.86 (2)
	10 PFC	3.73 (7)	0.91 (2)	1.65	0.94	2.53 (6)	0.95 (2)
	8 I [2]	1.60 (7)	0.45 (2)	4.10	0.29	2.15 (6)	0.21 (2)
	9 I	2.46 (7)	0.59 (2)	3.50	0.65	2.88 (6)	0.65 (2)
400 r	8 PFC	2.33 (5)	0.43 (4)	2.14	0.36	2.38 (4)	0.56 (2)
	9 PFC	1.76 (4)	0.48 (4)	2.54	0.35	2.30 (4)	0.41 (2)
	7 I	3.18 (5)	0.98 (4)	13.45	0.88	4.26 (4)	1.18 (2)
	10 I	2.46 (5)	0.93 (4)	5.91	0.61	2.45 (4)	0.83 (2)
650 r	32 PFC	2.52 (6)	0.80 (3)	1.58	0.78	2.55 (5)	0.82 (2)
	33 PFC	2.52 (5)	0.75 (3)	2.25	0.61	2.19 (5)	0.76 (2)
	35 PFC	2.32 (5)	0.76 (3)	1.96	0.68	3.20 (5)	0.91 (2)
	30 I	2.82 (5)	0.64 (3)	5.71	0.71	4.46 (5)	0.75 (2)
	31 I	3.01 (5)	0.93 (3)	5.48	0.92	3.09 (5)	1.07 (2)
	34 I	2.35 (5)	0.26 (3)	1.46	0.31	2.22 (5)	0.31 (2)

[1] The first day after irradiation included.

[2] I = irradiated. PFC = pair-fed control.

[3] The figures in parentheses indicate the number of daily determinations from which each mean was calculated.

treatment excretions of the irradiated monkeys and of the amounts excreted by the pair-fed controls.

As shown in table 3 in 6 of the 7 animals treated there was a post-irradiational increase in the urinary excretion of total ascorbic acid at least on the first day after exposure. The magnitude of the loss was less striking than was the

TABLE 4

Mean daily urinary excretion of pteroylglutamic acid by irradiated monkeys

IRRADIATION DOSE	ANIMAL NO.	DAYS PRIOR TO IRRADIATION	FIRST DAY AFTER IRRADIATION	ALL DAYS AFTER IRRADIATION [1]
		μg/day	*μg/day*	*μg/day*
325 r	7 PFC [2]	6.45 (5) [3]	4.70	1.31 (4)
	10 PFC	8.06 (4)	1.69	2.89 (4)
	8 I [2]	16.41 (5)	23.68	5.24 (4)
	9 I	11.44 (5)	20.73	3.33 (4)
400 r	8 PFC	25.90 (4)	14.85	11.88 (4)
	9 PFC	12.38 (4)	2.68	4.54 (4)
	7 I	8.38 (5)	21.30	3.14 (4)
	10 I	8.56 (5)	16.62	4.19 (4)
650 r	32 PFC	5.78 (5)	0.39	0.55 (4)
	33 PFC	6.20 (5)	3.68	1.50 (4)
	35 PFC	0.81 (5)	0.16	0.32 (4)
	30 I	2.23 (5)	8.90	0.68 (5)
	31 I	4.73 (5)	2.51	0.48 (4)
	34 I	0.92 (5)	0.28	1.87 (5)

[1] First day after irradiation included.

[2] I = irradiated. PFC = pair-fed control.

[3] The figures in parentheses indicate the number of daily determinations from which each mean was calculated.

enhanced excretion of diketogulonic acid. Serum ascorbic acid remained within the normal range following irradiation. It should be noted that this was the only vitamin fed separately as a tablet. It was usually consumed even when the animals were suffering from severe anorexia. This relatively constant intake may account in part for the lack of an effect of anorexia on the measurements. As previously noted (Dutra de Oliveira et al., '56) the urinary loss of ascorbic acid is

surprisingly low in view of the regular daily intake of 50 mg of this vitamin.

The excretion of pteroylglutamic acid and of vitamin B_6 varied from day to day and from animal to animal (tables 4 and 5). There was an increased urinary loss of both substances especially on the first day after irradiation when the monkeys were exposed to 325 r and 400 r, but this increase

TABLE 5

Mean daily urinary excretion of pyridoxine by irradiated monkeys

IRRADIATION DOSE	ANIMAL NO.	DAYS PRIOR TO IRRADIATION	FIRST DAY AFTER IRRADIATION	ALL DAYS AFTER IRRADIATION [1]
		mg/day	*mg/day*	*mg/day*
325 r	7 PFC [2]	0.024 (4) [3]	0.29	0.08 (4)
	10 PFC	0.033 (4)	0.09	0.16 (4)
	8 I [2]	0.020 (5)	2.00	0.16 (4)
	9 I	0.026 (5)	1.35	0.14 (4)
400 r	8 PFC	0.044 (4)	0.17	0.18 (4)
	9 PFC	0.048 (4)	0.09	0.17 (4)
	7 I	0.045 (5)	1.85	0.17 (4)
	10 I	0.017 (5)	0.85	0.10 (4)
650 r	32 PFC	0.012 (5)	0.08	0.12 (5)
	33 PFC	0.016 (5)	0.16	0.10 (5)
	35 PFC	0.013 (5)	0.11	0.11 (5)
	30 I	0.015 (5)	0.53	0.10 (4)
	31 I	0.012 (5)	0.21	0.10 (5)
	34 I	0.009 (5)	0.10	0.11 (5)

[1] First day after irradiation included.

[2] I = irradiated. PFC = pair-fed control.

[3] The figures in parentheses indicate the number of daily determinations from which each mean was calculated.

was not evident at 650 r. In neither instance was the loss as great as had been observed in rats following x-irradiation as shown by Pearson et al. ('53).

DISCUSSION

The clinical course of our monkeys exposed to x-irradiation was similar to that described by Eldred and Trowbridge ('54) who studied irradiation sickness in this species.

The monkey has a greater dependence upon dietary sources of pteroylglutamic acid and ascorbic acid than does the rat. Indeed, all the nutrients studied here are dietary essentials for the monkey. The less striking effect of irradiation on vitamin loss by the monkey than by the rat may be related to the differences in dietary requirement of the two species. It is pertinent to note that the increased loss by the monkey which followed irradiation at the levels employed was of temporary duration and was not of a magnitude indicative of a depleting phenomenon. The most pronounced increased excretion occurred on the first post-irradiation day, a timing which is compatible with the interpretation that the loss was due either to the general stress phenomenon or to destruction of susceptible tissue by the ionizing irradiation.

The decreased excretion of thiamine, riboflavin and N^1-methylnicotinamide following irradiation is not related to impaired absorption or excessive utilization. As is apparent from table 1, the decrease occurred both in the experimental and pair-fed control animals and is, therefore, related to restriction of food. The decrease in excretion is more pronounced and persistent when the food intake is restricted for several days, as was the case of animals receiving 650 r of irradiation. Such observations emphasize the necessity of pair-feeding in order to interpret experimental results obtained in studies of irradiation effects.

SUMMARY

Ten monkeys *(Macaca mulatta)* were exposed to three levels (325 r, 400 r, 650 r) of x-irradiation. Anorexia, leucopenia, loss of hair and at the high levels of irradiation, hemorrhage and diarrhea, followed.

Observations on pair-fed non-irradiated controls demonstrated that the decreased urinary excretion of riboflavin, thiamine and N^1-methylnicotinamide which followed irradiation was a result of food restriction, not a specific effect of the x-ray injury.

No meaningful changes could be found in blood ascorbic acid at the levels of irradiation and intake. Diketogulonic acid excretion was increased in urine after irradiation.

A temporary, moderately increased excretion of pteroylglutamic acid and of vitamin B_6 followed x-irradiation.

No prolonged loss of ascorbic acid, riboflavin, thiamine, niacin (N^1-methylnicotinamide), pyridoxine, or pteroylglutamate occurs which might indicate that x-irradiation is a conditioning agent in producing a deficiency of these factors.

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MOLYBDENUM DEFICIENCY AND TUNGSTATE INHIBITION STUDIES [1]

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The need for dietary molybdenum in order to establish normal levels of xanthine oxidase (XO) in rat tissues, especially intestine, has been demonstrated (Westerfeld and Richert, '54a; Richert and Westerfeld, '53; DeRenzo et al., '53). The presence of molybdenum in XO (Avis et al., '56; Kielley, '55; Remy et al., '55) and aldehyde oxidase (Mahler et al., '54), and the association of enzyme activity with its molybdenum content have also been established (Westerfeld et al., '56). These facts argue for an essential role of dietary molybdenum in animal nutrition. However, it is not yet known that the molybdenum-containing enzymes are necessary for the well-being or normal physiology of the animal, since from two-thirds to three-fourths of the total XO can be removed from the rat without altering its excretion of uric acid and allantoin (Bass et al., '50).

In approaching the problem of demonstrating an absolute requirement for dietary molybdenum in animal nutrition, three possibilities were apparent: (1) The preparation and use of a molybdenum-free diet, (2) the preliminary depletion of molybdenum from tissues by feeding a low-protein diet (Westerfeld and Richert, '54b), and (3) the use of sodium tungstate (Na_2WO_4) as a competitive inhibitor of molyb-

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denum (DeRenzo, '54). All three approaches were studied. Both rats and chicks were used — the former because of the availability of much pertinent background information, and the latter because of the possibility of imposing the dietary restriction from birth rather than weaning, the low molybdenum content of eggs (Westerfeld and Richert, '53), the rapid growth rate of young chicks, and the presumed need for liberal quantities of xanthine dehydrogenase in bird tissues in order to form the large quantities of uric acid produced by this species. Criteria studied were growth rate, reproduction, purine oxidation and tissue levels of XO and molybdenum. Molybdenum and tungsten were added to all diets as Na_2MoO_4 and Na_2WO_4 but concentrations were recorded for the element alone.

PROCEDURE

Molybdenum-poor diets

The purified 24% casein diet previously used (Westerfeld and Richert, '54a) for demonstrating the molybdenum effect on intestinal XO (Richert and Westerfeld, '51a) contained approximately 20 μg Mo/kg by either microbiological (Nicholas, '52) or chemical assay (Westerfeld and Richert, '53). The casein [2] contained 3 to 4 μg, glucose 1 μg, and the salt mixture [3] 1 to 3 μg/100 gm; these constituents accounted for 15 to 17 of the 21 μg Mo/kg diet actually found — the oils presumably contributing the difference. The intestinal XO assay curve (Westerfeld and Richert, '54a) appears to be an accurate indicator of available dietary molybdenum, since an extrapolation of this curve to zero activity (as determined with methylene blue) intersects the abscissa at approximately — 20 μg Mo/kg diet.

In previous studies with weanling rats this diet gave no indication of a molybdenum deficiency other than its effect on intestinal XO. For the purpose of raising several gen-

[2] Labco.
[3] Phillips and Hart ('35).

erations of rats this diet was supplemented with 2 gm of mixed tocopherols (34%), 1 gm inositol, 100 mg ascorbic acid, 10 mg menadione and biotin, 2 mg folic acid and 0.5 μg vitamin B_{12}/kg. Sprague-Dawley female weanling rats were raised and maintained on this diet, except for a two-week period starting 5 weeks before mating they were fed a protein-free diet (replacing the casein with glucose) in order to deplete the liver of molybdenum. Second generation litters were brought to maturity on this diet and mated in the same way in order to produce further generations.

Third generation weanling male rats three to 4 weeks old were divided into three groups: (1) continuation of the same diet, (2) the diet supplemented with 200 μg Mo/kg, and (3) the diet containing 7.2 mg Na_2WO_4 (4.5 mg W)/kg. During the 7 weeks these diets were fed, there was no difference in the growth rates of the rats in any of the three groups. Tissue XO was determined manometrically (Westerfeld and Richert, '52) and liver molybdenum was measured chemically (Westerfeld and Richert, '53) at this point, and the results are given in table 1; corresponding values of the second generation rats obtained at weaning were identical with those of subsequent generations and are also shown in table 1. By comparison with rats fed a commercial dog chow [4] (Westerfeld and Richert, '49, '54a; Richert and Westerfeld, '51b), tissue XO levels in these rats were sharply reduced by this dietary regime. However, the growing rat was capable of abstracting sufficient molybdenum from this diet to deposit relatively normal amounts of XO in all tissues except intestine. The addition of 4.5 mg of W/kg of diet prevented this build-up of tissue XO and liver molybdenum much beyond the starting weanling level, and increased the urinary excretion of molybdenum from less than 0.07 μg/day/6 rats to about 0.6 μg. No other effects from the tungstate were noted.

The uric acid (Folin, '22) and allantoin (Young and Conway, '42) excretions of second or third generation weanling rats were similar to chow-fed controls. In addition the intra-

[4] Purina.

peritoneal injection of 37.5 mg xanthine/100 gm rat in an xanthine load test (Williams et al., '50) with weanlings, with or without tungstate feeding for 9 days, did not elicit any defect in uric acid-allantoin formation. About 50% of the administered xanthine was excreted as urinary allantoin (16 mg) and uric acid (1 mg); no significant increase occurred in urinary xanthine (0.2 mg). The tissue XO levels in the tungstate-fed rats were approximately equal to those of the molybdenum-poor weanlings of table 1; liver molybdenum was 0.32 μg/gm dry weight. Rats fed the purified diet alone for the 9 days of the test had levels of tissue XO which were increased significantly above the weanling level; other rats analyzed at various times after weaning also showed a relatively rapid build-up of tissue XO on the purified diet.

The feeding of a similar diet (but containing 18% casein, 10% gelatin and 0.3% L-cystine) to one-day-old chicks for 6 to 7 weeks also failed to produce a molybdenum-deficiency in this species. By comparison with a control group of chicks fed the same diet supplemented with 2 mg Mo/kg, the molybdenum-poor diet containing approximately 40 μg Mo/kg had no effect on growth rate (11.5 gm/day), xanthine dehydrogenase activities in liver, kidney, or intestine (35, 28, and 12 mm^3 O_2/20 min. respectively in the presence of methylene blue), or liver molybdenum (2.7 μg/gm dry weight). Since livers from the day-old chicks at the start of the experiment contained 1.22 μg Mo/gm dry weight, the chicks had no difficulty in obtaining adequate amounts of molybdenum from the molybdenum-poor diet.

The molybdenum content of the rat diet could be reduced to approximately 10 μg/kg by purifying the glucose in solution with an anion exchange resin [5] and by leaching the casein with hot water. No extensive studies with this diet were conducted, since (1) the experiments with protein-deficient diets suggested that still lower levels of dietary molybdenum

[5] Amberlite IRA-410.

TABLE 1

Xanthine oxidase activities and liver molybdenum in third generation rats raised on a molybdenum-poor diet as compared with similar rats fed Na_2MoO_4 or Na_2WO_4 for 7 weeks

DIET	BODY WEIGHT	NO. OF RATS	XANTHINE OXIDASE ACTIVITY (DETERMINED WITH METHYLENE BLUE)					LIVER MOLYBDENUM
			Liver	Intestine	Kidney	Spleen	Lung	
	gm		*mm^3 O_2/20 min/283 mg fresh tissue*					*μg/gm dry wt.*
Mo-Poor [1]	35	8	3 ± 1.0	4.4 ± 0.6	0.8 ± 0.5	0	2.1 ± 0	0.82 ± 0.13
Mo-Poor	197	11	32 ± 1.8	16 ± 2.1	4 ± 0.4	12 ± 1.5	11 ± 0.9	1.89 ± 0.15
+ Mo	212	6	37 ± 4.7	42 ± 2.2	6 ± 1.8	11 ± 1.5	11 ± 1.8	2.24 ± 0.19
+ W	200	6	17 ± 3.8	2 ± 0.8	0.8 ± 0.4	6.5 ± 1.7	5.3 ± 0.5	0.81 ± 0.18

[1] Weanlings analyzed at three to 4 weeks of age.
[2] Mean ± standard error.

TABLE 2

The effect of feeding a low-molybdenum protein-free diet for two to three weeks on the concentration of molybdenum in adult rat tissues. All values expressed as μg Mo per gm dry weight ± S.E.

DIET	LIVER	KIDNEY	STOMACH	INTESTINE	COLON	SPLEEN	LUNG	BRAIN	SKIN	MUSCLE
Chow [1]	1.84	1.04	0.40	0.53	0.24	0.52	0.37	0.24	0.15	0.06
	± .06 [2]	± .12	± .02	± .08	± .03	± .06	± .03	± .03	± .02	± .01
Protein-free	0.67	0.77	0.32	0.40	0.26	0.57	0.36	0.14	0.15	0.03

[1] Hair contained about the same molybdenum concentration as skin; blood molybdenum was too low to be detected (< 0.05 μg/gm dry weight).
[2] Mean ± standard error.

would probably be required in order to produce a deficiency, and (2) tungstate inhibition offered a more promising approach.

Low-protein diets

Tissues from groups of approximately 20 adult hooded rats were pooled, dried at 80° and analyzed chemically for molybdenum. Control rats were fed a laboratory chow[6]; others were fed a purified protein-free diet (Westerfeld and Richert, '54a) containing approximately 15 μg Mo/kg for two to three weeks before analysis. Table 2 shows the effect of feeding a low-molybdenum protein-free diet on the removal of molybdenum from the tissues. Most tissues lost some molybdenum but the major effect was on the liver. Feeding a purified low-molybdenum, 24% casein diet to adult rats for two weeks did not decrease the liver molybdenum appreciably (1.58 μg/gm), while feeding such a diet after protein depletion restored the liver molybdenum to 1.4 μg/gm. The addition of 1 mg Mo/kg to the 24% casein and protein-free diets gave liver molybdenum values of 2.1 and 1.05 μg/gm respectively. While the molybdenum content of the diet had some effect on the concentration of molybdenum in the liver, the dominant factor was dietary protein.

From the values in table 2, it can be estimated that the normal 200 gm rat contained a total of 8 to 10 μg/Mo, about half of which was in the liver. As a result of protein depletion, the total body molybdenum was reduced to 4 or 5 μg. Since protein depletion did not interfere with the formation of uric acid and allantoin (Bass et al., '50), this presumed function of molybdenum was fulfilled by this small amount of body molybdenum. It seems evident that a "molybdenum-deficient" diet will have to be exceedingly low in molybdenum in order to prevent the rat from receiving the few micrograms of molybdenum known to be adequate.

From 50 to 85% (average of 75%) of the 2 to 3 μg Mo/gm dry weight found in a normal chow-fed rat liver is present

[6] See footnote 4, page 541.

in the combined precipitate of nuclei, mitochondria and microsomes (obtained by centrifuging a phosphate buffer homogenate for three hours at 30,000 × gravity, resuspending, and centrifuging for two hours). After 25 days on a protein-free diet, the liver contained 0.8 μg Mo/gm dry weight, of which two-thirds was in the combined precipitate and one-third in the supernatant. Since XO accounts for less than 10% of the total liver molybdenum, is localized in the supernatant fraction, and is removed from the liver by a low-protein diet, it is evident that other molybdenum-proteins whose functions are unknown may be of greater importance than XO to the life of an animal.

TABLE 3

Composition of basal chick diet

CONSTITUENT	AMOUNT	CONSTITUENT	AMOUNT
	gm/100 gm		*mg/100 gm*
Glucose	59	Mixed tocopherols (34%)	200
Labco casein	22	Choline chloride	200
Phillip-Hart Salts	4.3	Inositol	100
Cellulose	4	Para-aminobenzoic acid	5
Crisco	4	Ca pantothenate	3
Wesson oil	2	Niacin	3
Cod liver oil	1	Pyridoxine	0.5
Glycine	2.1	Biotin	0.03
L-arginine mono-HCl	0.9	Riboflavin	0.5
L-Cystine	0.6	Folic acid	0.4
		Thiamine chloride	0.3
		Menadione	0.2
		Vitamin B_{12}	0.003

Urinary excretion of molybdenum. Figure 1 shows the influence of various diets on the urinary excretion of molybdenum by adult rats. When the protein was removed from the usual 24% casein diet by replacing it with an equal quantity of glucose, there was a temporary small spike in the excretion of molybdenum as the latter was removed from the tissues. When the protein was removed from a diet containing 100 or 200 μg of added Mo/kg, the urinary excretion of

molybdenum was depressed slightly from the levels obtained with similar diets containing 24% casein. However, there was no gross impairment in the absorption of Na_2MoO_4 from protein-free diets. With liver residue supplying the dietary molybdenum, there was a slightly greater excretion of urinary molybdenum when the 21% casein was removed from the diet

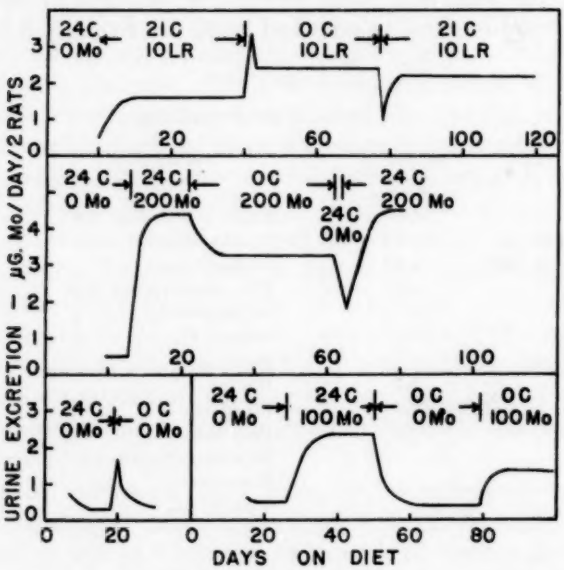

Fig. 1 The effect of diets containing varying amounts of casein (in %), liver residue (in %) and Mo (µg Mo/kg, fed as Na_2MoO_4) on the urinary excretion of molybdenum.

than when it was present. This may be related to a better utilization of the liver residue when the diet is otherwise protein-free, so that more of the bound molybdenum in the liver residue becomes available (Westerfeld and Richert, '53). It is evident that molybdenum absorption is not dependent upon dietary protein in the same way that liver XO is related to the protein intake.

Tungstate inhibition

DeRenzo ('54) originally reported an inhibition of the intestinal XO response to molybdenum by feeding tungstate, and Pinsent ('54) found a competitive relationship between tungstate and molybdate with *E. coli.* This inhibition was therefore studied in the usual bioassay procedure for molybdenum (Richert and Westerfeld, '51a). Male albino weanling rats were fed the purified 24% casein diet for 6 to 8 days to deplete the intestine of XO. For 7 days, groups of 6 rats were then fed the same diet to which were added varying amounts of sodium molybdate and increasing amounts of sodium tungstate (as shown with the results). The resulting intestinal XO values are given in figure 2 and show that a dietary level of tungsten equivalent to 100 times the molybdenum content completely inhibited the deposition of intes-

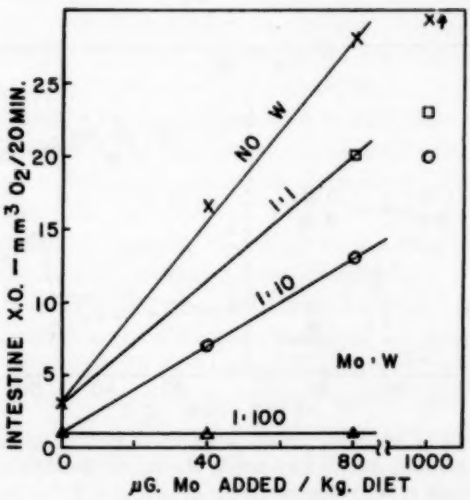

Fig. 2 The effect of feeding sodium tungstate, at the indicated molar ratios, on the intestinal xanthine oxidase response to sodium molybdate in the bioassay procedure for the xanthine oxidase factor.

tinal XO. Smaller proportions of tungstate were progressively less effective, but produced an easily measurable effect.

Tungstate not only interfered with the deposition of intestinal XO, but actually displaced molybdenum from the tissues. The daily urine and feces from 12 adult hooded stock rats (375 gm) in 6 metabolism cages were analyzed for molybdenum as individual or pooled samples when necessary. After an additional week on the usual chow diet (1.08 μg Mo/gm), the rats were fed the purified 24% casein diet containing

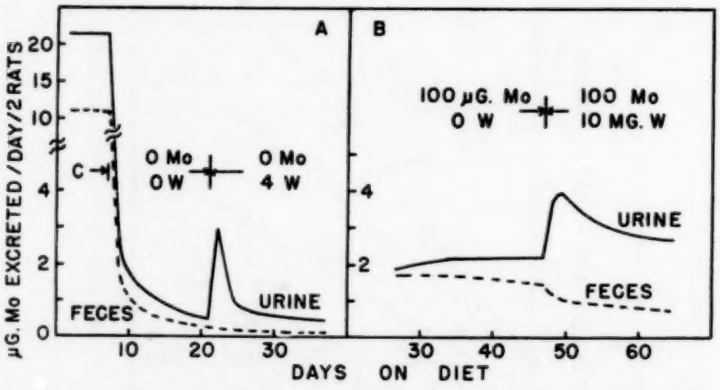

Fig. 3 The effect of adding sodium tungstate to 24% casein diets containing: (A) no added molybdenum, or (B) 100 μg Mo/kg (as Na_2MoO_4) on the urinary and fecal excretion of molybdenum.

C represents a chow diet. Values for tungstate are in mg W/kg diet and represent a Mo: W ratio of 1: 100 with the molybdenum present or added to the purified diet.

approximately 21 μg Mo/kg for two weeks; 4.04 mg W/kg diet were then added to give a W : Mo ratio of 100 : 1. Figure 3 A shows the urinary and fecal excretion of molybdenum in relation to the diet fed. On the purified diet the urinary excretion fell from the average level in chow-fed rats of 10.8 μg Mo/rat/day to about 0.25 μg within two weeks; similarly the fecal excretion fell from 5.6 to 0.1 μg Mo. The addition of Na_2WO_4 to the purified diet caused a sharp spike in the urinary molybdenum excretion. The effect was relatively

short-lived, but it was estimated that approximately one-half of the molybdenum present in the rat tissues was removed by the tungstate feeding.

Figure 3 B shows that the presence of tungsten in the diet did not inhibit the absorption of molybdenum from the diet. Twelve adult rats were fed a 24% casein diet containing an added 100 μg Mo/kg for approximately 7 weeks. Excretions of molybdenum in urine and feces were measured during the last three weeks. Ten milligrams of tungsten (16 mg Na_2WO_4)/kg were then added to the diet (to give a W : Mo ratio of 100 : 1). Urinary molybdenum showed the spike in excretion as molybdenum was removed from the tissues, and there continued to be a large amount of molybdenum excreted in the urine during the remaining three weeks of the experiment. Fecal molybdenum actually decreased after the addition of tungsten to the diet.

After 4 weeks of tungstate feeding to the rats in figure 3 A, no xanthine or hypoxanthine was detected in the urine by using the latter as a potential substrate for XO; hypoxanthine added to the urine in vitro was oxidized at a normal rate. Hydrolysis of the urine by heating with 1 N HCl for one hour also did not release anything which would give an oxygen uptake with milk XO. While some of the tissues of these adult rats were partially depleted of XO by the tungstate feeding at a level of 100 : 1 with molybdenum, there were adequate amounts of the enzyme still present. The XO activities (determined in the presence of methylene blue) of the spleen, lung, liver, kidney and intestine were 13 ± 0.84, 10 ± 0.57, 23 ± 2.74, 4 ± 0.52 and 3 ± 0.64 mm³ O_2/20 min./283 mg tissue respectively, as compared with corresponding values of 13, 11, 65, 8 and 60 for normal chow-fed rats (Westerfeld and Richert, '49, '54a; Richert and Westerfeld, '51b). The molybdenum content of the liver was 0.66 μg/gm dry weight.

Molybdenum-deficiency by tungstate-inhibition

Experiments with chicks. The addition of sodium tungstate to the diet at 1000 and 2000 times the estimated molybdenum

content produced what appeared to be a molybdenum deficiency in chicks, since the effects were overcome by adding more molybdenum to the diet. The diet is given in table 3.

White Leghorn day-old chicks were divided into 9 groups which were fed the above diet with varying amounts of tungsten and molybdenum, as shown with the results in table 4. Diets containing 45 or 94 mg W/kg (groups 4 and 8) reduced the growth rate somewhat, and this inhibition was largely overcome by 2, 20 or 60 mg of added molybdenum per kilogram (groups 5, 6, 7, 9). Deaths occurred only in groups 4, 8 and 9, in which the mortalities were 14, 24 and 9% respectively at three weeks. After 5 weeks, the mortality was 24% in group 4, 9% in group 5, 28% in group 8, 9% in group 9, and zero in all others.

After three weeks on the diets, 6 chicks each from groups 8 and 9 were sacrificed, and analyzed for xanthine dehydrogenase (Remy et al., '51). The mean values for intestine, liver, kidney and pancreas were 0.4, 2.2, 0.2 and 0.8 mm^3 $O_2/20$ min. respectively for the chicks of group 8, while the corresponding values for those of group 9 were 7.8, 14.4, 10.1 and 2.9. The molybdenum contents of the livers were 0.20 and 3.1 μg/gm dry weight respectively for the two groups. Thus within three weeks the addition of 94 mg W/kg to the diet markedly depressed the tissue molybdenum and xanthine dehydrogenase activities, and this effect was reversed or prevented by the simultaneous addition of 60 mg of molybdenum per kilogram.

After 6 weeks on the diet, 8 chicks from each group were analyzed for xanthine dehydrogenase (XD), while the tissues from the remaining chicks were pooled for molybdenum determinations. Enzyme activities (shown in table 4) were markedly depressed by the tungstate in all tissues known to contain XD (Remy et al., '51), but were completely restored by all levels of molybdenum added. Tissue molybdenum values (recorded in table 5) were also markedly reduced by the tungstate in group 4; insufficient tissues were available from the chicks of group 8 for the molybdenum analyses. The addition

TABLE 4

The effect of diets containing varying amounts of tungsten (as Na_2WO_4) and molybdenum (as Na_2MoO_4) on growth and tissue xanthine dehydrogenase activities of chicks

GROUP	ADDITIONS TO DIET		BODY WEIGHT [2]		XANTHINE DEHYDROGENASE ACTIVITIES			
	W	Mo	At 3 wks.	At 5 wks.	Intestine	Liver	Kidney	Pancreas
	mg/kg	*mg/kg*	*gm*	*gm*		*mm³ O_2/20 min/flask* [3]		
1 [1]	..	..	218	343	8	32	11	6
2	..	.. [1]	217	311	8	32	12	4
3	..	0.2	203	339	5	27	11	5
4	45	..	169	311	1	4	1	1
5	45	2	196	339	7	34	11	4
6	45	20	206	332	7	35	17	5
7	45	60	186	322	6	29	14	7
8	94	..	149	274	1	3	2	1
9	94	60	177	324	5	29	12	5

[1] Group 1 was fed Park and Pollard starter mash; group 2 was fed the synthetic diet to which no molybdenum was added, but which contained approximately 0.02 mg/kg Mo.

[2] Starting weights averaged 40 to 44 gm for all groups. Twenty-nine chicks per group for groups 4 and 8; 22 chicks per group for all others.

[3] XD values were determined on 8 chicks from each group after 6 weeks on the diets, using 28.3 mg of liver or kidney and 84.9 mg of intestine or pancreas per flask.

TABLE 5

The molybdenum content of chicken tissues after 6 weeks on diets containing varying amounts of tungsten and molybdenum
(Same diet groups as in table 4)

DIET GROUP	1	2	3	4	5	6	7	8	9
					μg Mo/gm dry wt.				
Proventriculus	0.21	0.15	0.18	0	0.19	0.83	1.51	...	6.79
Intestine and colon	0.51	0.24	0.35	0.05	0.76	1.11	1.82	...	> 5.75
Kidney	4.44	3.09	3.48	0	1.65	6.85	> 8.80	...	14.45
Brain	...	...	0.17	0	0.05	0.12	0.59	...	0.82
Skeletal muscle	0.14	...	0.10	0.08	0.10	0.44	1.07	...	0.77
Liver (± S.E.)	3.56 ± .28	2.52 ± .12	3.35 ± .16	0.22 ± .04	2.73 ± .15	3.55 ± .25	4.43 ± .40	0.20 ± .01	4.11 ± .22

of molybdenum to the tungstate diets restored these levels to normal (or above, when the molybdenum content of the diet was high). The maximum effect of tungstate on liver molybdenum was already evident at three weeks.

Hemoglobin levels in the blood obtained at sacrifice were unaffected by the various diets.

Purine excretions. After three weeks on the experimental diets the uric acid excretion by chicks of group 8 had dropped to one-half that in group 9, as determined by the Folin silver precipitation method. An extract of the excreta from chicks of group 8 incubated with purified milk XO gave an oxygen uptake of 21 micromoles per 100 mg, which was equivalent to a concentration of 3.1% xanthine or 1.6% hypoxanthine in the original dry excreta. There was no oxygen uptake by the extract from chicks of group 9 treated in the same fashion.

After 6 weeks on the diets and at the time the chicks were sacrificed, the pooled cloacal contents of each group were obtained, dried at 75°C. and then in a desiccator, and analyzed as follows: 100 mg of the dry material was extracted with 5, 3 and 2 ml portions of warm 0.1 N NaOH over a period of one hour. Uric acid was determined on a silver precipitate of the combined extracts according to the method of Folin ('22). The purine components were identified by paper chromatography, using a solvent consisting of 77% n-butanol, 13% H_2O and 10% formic acid (Markham and Smith, '49). Aliquots of the extracts were treated with enough purified milk XO to convert all of the xanthine and hypoxanthine to uric acid; the amount of oxygen used was determined manometrically, the amount of uric acid formed was measured colorimetrically, and the relative amounts of hypoxanthine and xanthine present were then calculated. In addition, the xanthine separated by paper chromatography was eluted from the paper with 0.1 N NaOH and measured quantitatively by the procedure of Williams ('50).

The excreta from all groups except 4 and 8 contained from 15 to 27% uric acid (av. = 20.6), and no xanthine or hypoxanthine could be detected with milk XO or by paper chro-

matography. The excreta from groups 4 and 8 contained only 11.8 and 10.86% uric acid respectively, and both samples contained hypoxanthine and xanthine as demonstrated by the combination of chromatography and the action of XO. The excreta from group 8 consumed 79 micromoles of O_2/100 mg with milk XO, and formed an additional 8.74 mg of uric acid by both colorimetric and spectrophotometric measurements. These results indicated the presence of 4.49% of xanthine and 3.15% hypoxanthine in the dry material. The xanthine determination on the paper chromatogram eluate gave a value of 5.05%. On a molar basis, the chicks of group 8 excreted 54% of the purine mixture as uric acid, 26% as xanthine and 20% as hypoxanthine.

Experiments with rats. Experiments similar to those just described for chicks were also conducted with 9 groups of 18 Sprague-Dawley male weanling rats each. The diet used was the 24% casein diet, supplemented as previously described for raising several generations of rats; Na_2WO_4 and Na_2MoO_4 were added to the various groups as described for the chick diets.

During the 12 weeks that the rats were maintained on these diets, the tissue levels of molybdenum and XO in the tungstate-fed rats were markedly depleted, but there were no other indications of a molybdenum deficiency. All control and experimental groups showed comparable weight gains over the 12-week period, and there were no deaths in any group. The urinary excretions of uric acid, allantoin and xanthine, determined at two-week intervals and expressed as creatinine ratios (Bass and Place, '49), were essentially the same in all groups. The rats in group 8 excreted 0.42 mg of xanthine per day as compared with 0.1 mg for the control groups, but the allantoin and uric acid excretions were essentially the normal values of 34 and 3.3 mg/day/rat respectively. An xanthine load test conducted with groups 4 and 8 after 11 weeks on the diet gave the same excretion pattern obtained with normal controls; during the 12 hours following a 37 or 75 mg dose of xanthine intraperitoneally, the basal allan-

toin: creatinine and uric acid: creatinine ratios of 4 and 0.35 were increased two- to three-fold, while the basal xanthine: creatinine ratio of 0.02 was increased 6 to 10 times. Urines collected during the 12- to 24-hour period after xanthine administration had normal xanthine ratios, while the allantoin and uric acid excretions were still slightly elevated. All values in the 24- to 51-hour urines had returned to normal. Blood hemoglobin levels were the same on all diets.

Tissues from 6 rats were analyzed for XO after 6 weeks on the diets, and the tissues from the remaining rats were pooled for molybdenum analyses at the termination of the 12-week period. The results are shown in tables 6 and 7. As with the chicks, dietary tungstate markedly depleted the molybdenum and XO in all tissues of the rat, and the addition of molybdenum to the diet reversed these effects. In contrast to the chicks and in spite of the very marked depletion of tissue molybdenum and XO, the rats grew normally and continued to oxidize purine precursors to uric acid and allantoin without difficulty.

Other possible inhibitors

Sulfate had a marked effect on molybdenum metabolism in sheep (Dick, '56), but by comparison with tungstate had no effect on the deposition of intestinal XO in the rat. Albino weanling male rats were fed the 24% Labco casein diet for 6 to 9 days, then the same diet with varying amounts of added Na_2MoO_4 and Na_2SO_4 for 7 days before the tissues were analyzed for XO. With the control diet containing no added molybdenum, liver XO was 30 and 20 ($\pm$ methylene blue) mm^3 O_2/20 min./283 mg. The addition of 600 mg of Na_2SO_4 per kilogram of diet did not change these values. With 0, 40 and 80 μg of molybdenum added per kilogram of diet, intestinal XO values (no methylene blue) were 4, 13 and 29 respectively; the same values were obtained when the diets also contained 600, 600 and 1200 mg/kg of Na_2SO_4 respectively (for a ratio of 10,000:1 of added sulfur and molybdenum).

A similar experiment with Na_2CrO_4 showed no effect of chromate on molybdenum metabolism in the rat. With the control diet containing no added molybdenum, liver XO was 25 and 13 (± methylene blue); the addition of 3.37 mg Na_2CrO_4

TABLE 6

The effect of diets containing varying amounts of tungsten (as Na_2WO_4) and molybdenum (as Na_2MoO_4) on tissue xanthine oxidase activities and liver molybdenum of weanling rats fed for 6 weeks

GROUP	ADDITIONS TO DIET		XANTHINE OXIDASE ACTIVITIES (WITH METHYLENE BLUE)					LIVER MOLYBDENUM
	W	Mo	Intest.	Liver	Kidney	Lung	Spleen	
	mg/kg	*mg/kg*	*mm^3 O_2/20 min/283 mg fresh tissue*					*µg/gm dry tissue*
1 [1]	..	..	30	31	9	10	10	1.72
2	..	.. [1]	7	28	5	11	10	0.85
3	..	0.2	33	35	9	11	12	1.53
4	45	..	1	1	1	1	0	0.15
5	45	2	23	31	10	12	13	0.72
6	45	20	44	37	8	15	18	1.77
7	45	60	29	38	8	11	16	2.27
8	94	..	1	1	1	0	0	0.11
9	94	60	35	38	6	11	16	2.13

[1] Group 1 was fed chow; group 2 was fed the synthetic diet to which no molybdenum was added but which contained approximately 0.02 mg Mo/kg.

TABLE 7

The molybdenum content of rat tissues after 12 weeks on diets containing varying amounts of tungsten and molybdenum

(Same diet groups as in table 6)

DIET GROUP	1	2	3	4	5	6	7	8	9
	µg Mo/gm dry wt.								
G.I. tract [1]	0.25	0.17	0.19	...	0.31	0.79	0.84	0	> 4.06
Kidney	1.31	0.72	1.20	0.18	0.92	4.62	> 9.55	< 0.23	> 10.3
Lung and spleen [2]	0.36	...	...	0.10	0.38	1.49	> 5.84	0.03	4.43
Skeletal muscle	...	0.06	0.04	0.02	0.02	0.19	0.77	0.01	> 1.42
Liver	1.64	0.91	1.56	0.17	1.00	1.98	2.86	0.09	2.25
(± S.E.)	± .08	± .02	± .09	± .02	± .18	± .10	± .17	± .00	± .37

[1] The entire stomach, small intestine and colon were pooled to provide sufficient material for analysis.

[2] The lungs and spleen were pooled for molybdenum analysis.

per kilogram of diet did not change these values. With 0, 40, 80 and 1000 μg molybdenum added per kilogram diet, intestinal XO values (no methylene blue) were 3, 10, 19 and 22 respectively; the same values were obtained when the diets also contained 3.37, 6.75, 13.5 and 169 mg/kg of Na_2CrO_4 respectively (100:1 ratio of Cr:Mo). There was also no depression of growth rate by the chromate.

DISCUSSION

The rat has a remarkably low requirement for molybdenum and tissue XO. In spite of the virtual elimination of these constituents from all tissues by feeding tungstate, no overt effects of such deficiencies were evident in the intact animal. The normal rat clearly has a tremendous reserve of molybdenum and XO. The occurrence of more obvious deficiency symptoms in the chick under similar dietary conditions may be attributable to a greater need by the chick for molybdenum and xanthine dehydrogenase in the formation of large amounts of uric acid by this species. The deficient chick still excreted half of the daily purine output as uric acid; the actual amount of uric acid formed still greatly exceeded the amount of xanthine presented to the rat in the largest load test. If the rat had as large a requirement for xanthine oxidation as the chick, it too might have developed overt deficiency symptoms. The largest amount of sodium tungstate used in these studies (94 mg W/kg) was approximately one-tenth the dietary level found by Kinard and Van de Erve ('41) to give minimal toxicity symptoms in young rats.

SUMMARY

Rats maintained on a purified diet containing approximately 20 μg Mo/kg grew at a normal rate, reproduced, accumulated xanthine oxidase (XO) in tissues other than the intestine, and oxidized xanthine normally. Second and third

generation rats had very low levels of tissue XO at weaning, but these increased to relatively normal values on the diet.

Feeding a low-molybdenum protein-free diet to adult rats for two to three weeks had little or no effect on the molybdenum content of colon, spleen, lung or skin, but removed one-fourth of the molybdenum normally present in stomach, intestine and kidney, one-half of the molybdenum in muscle and brain, and two-thirds of molybdenum in liver. One-fourth of the molybdenum in normal liver and one-third of the molybdenum remaining after protein depletion was present in the supernatant fraction.

Dietary Na_2WO_4 equivalent to a W:Mo ratio of 100:1 completely inhibited the deposition of intestinal XO and markedly reduced the XO and molybdenum in rat liver. Rats fed Na_2WO_4 in a 1000 or 2000:1 ratio of W:Mo grew normally and oxidized xanthine to uric acid and allantoin as well as the controls in spite of the fact that all tissues were depleted of molybdenum and XO; the tissue changes were deversed by additional dietary Na_2MoO_4.

Chicks fed synthetic diet containing approximately 20 μg Mo/kg developed normally, but the addition of Na_2WO_4 at a level equivalent to a W:Mo ratio of 1000 or 2000:1 produced an apparent molybdenum deficiency. Growth rates were depressed somewhat, and mortality was approximately 25% in 5 weeks. All tissue xanthine dehydrogenase and molybdenum concentrations were markedly depleted by the tungstate, and about one-half of the uric acid normally excreted by chicks was replaced by a mixture of xanthine and hypoxanthine. All of the effects of the 1000:1 tungstate level were completely reversed by adding an additional 2 mg molybdenum, as Na_2MoO_4, per kilogram of diet.

The molybdenum removed from the tissues by protein deficiency or tungstate feeding appeared in the urine as a sharp spike in molybdenum excretion; neither diet interfered with the absorption of molybdenum from rat intestine. Neither Na_2SO_4 nor Na_2CrO_4 affected the metabolism of molybdenum.

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UTILIZATION OF CALCIUM, PHOSPHORUS, RIBOFLAVIN AND NITROGEN ON RESTRICTED AND SUPPLEMENTED DIETS[1]

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Allergic reactions in humans are frequently encountered with the use of such basic foods as milk, eggs and wheat. Many persons with food allergies must omit all three from the diet, while numerous adults consume little milk because of dislike or cost. Since the resulting diets probably furnish considerably less than the recommended amounts of calcium, phosphorus and riboflavin, a study of the adequacy of such restricted diets with and without mineral and vitamin supplements seemed of interest. In the present experiment utilization of calcium, phosphorus, riboflavin and nitrogen by young women was determined on (a) diets restricted in milk, eggs and wheat, (b) the restricted diets supplemented with minerals and vitamins, and (c) an adequate diet of natural foods including milk, eggs and wheat.

Dicalcium phosphate was chosen as the mineral supplement for several reasons: the salt is one frequently prescribed by physicians as a calcium supplement; the calcium-phosphorus ratio in this mineral is the same as that in milk, which made it seem an ideal supplement in an experiment where

[1] This paper is a contribution from the Southern Regional Nutritional Status Project S-15, Subproject 1: "Nutritional Requirements and Utilization."

equal amounts of calcium and phosphorus were to be supplied from mineral and food sources; and reports in the literature (Stearns and Jeans, '34–'35; Kempster et al., '40) indicated that the calcium from dicalcium phosphate was as well utilized by children as the calcium from milk.

EXPERIMENTAL

The experiment was designed to determine utilization by the same subjects of calcium, phosphorus and riboflavin on a diet supplying low levels of these nutrients and on the same diet when intake of the nutrients was raised to recommended levels or above by the addition of either $CaHPO_4$ and riboflavin or natural foods. Nitrogen utilization was determined also throughout the study. In the first year the restricted diet lacked only milk and milk products, in the second year milk and eggs were omitted, and in the third year milk, eggs and wheat were lacking.

During each year the procedure was essentially the same. A three-week period on the restricted diet preceded a 5-week period of supplementation of the restricted diet with $CaHPO_4$ and crystalline riboflavin. After a two-week interval on self-selected diets during the Christmas vacation, another three-week restricted period was followed by a 5-week period during which the natural foods which had been eliminated from the restricted diet were added in amounts to supply as nearly as possible the quantity of calcium, phosphorus and riboflavin previously given in the form of $CaHPO_4$ and the synthetic vitamin. During each period 4-day balance studies were made after one week and during the last 4 days; a third was made about the middle of the supplemented periods. During a preliminary experiment in the fall of 1950, 5 subjects remained on the restricted diet for 4 weeks, one balance study being made after one day on the diet and two others at weekly intervals. Since the pattern of excretion of calcium, phosphorus and riboflavin did not change, a three-week period of restriction appeared adequate. The experiment proper

began in January, following the plan already described except for omission of the first balance study in the final restricted period. This, together with the preliminary study, supplied data for 6 balance determinations on the restricted diet in 1950–'51 and only 4 in subsequent years.

The restricted diets were as nearly adequate as was possible when all dairy products, eggs and wheat were eliminated. A variety of other common foods supplied all nutrients except calcium, phosphorus and riboflavin at levels calculated (Watt and Merrill, '50) to meet recommended allowances. Average daily intakes of calcium, phosphorus and riboflavin were 264 to 358 mg, 785 to 885 mg and 0.99 to 1.47 mg respectively. Supplementation [2] of the restricted diets with $CaHPO_4$ raised the daily calcium intake to between 1.0 and 1.5 gm and in the diets of natural foods milk supplied equivalent amounts of calcium. The amount of synthetic riboflavin added to the restricted diets was equal to the estimated riboflavin content of the milk. In 1950–'51 the $CaHPO_4$ supplement accidentally furnished approximately one gram of calcium per day instead of the quantity to make a total daily intake of one gram, and almost 4 cups of milk were required to provide an equal amount of calcium. For this reason, the riboflavin level in the supplemented period was considerably below that in the corresponding period on the diet of natural foods. When milk, eggs and wheat were restored to the diet in the final periods, considerable decrease in the quantities of meat and other foods was necessary to keep the levels of protein and calories the same as before. All other nutrient levels were calculated to be unchanged except that niacin fell below the recommended allowance. Each year, therefore, a daily supplement of 5 mg of nicotinic acid was given throughout the final period. All diets furnished approximately 1900 calories, with sugar,

[2] In 1950–'51, the supplement was prepared by mixing the required amount of crystalline riboflavin with $CaHPO_4$ powder. The daily supplement for each subject was weighed and packaged in capsules which were taken at mealtime. In the following years Lilly's $CaHPO_4$ tablets were substituted, and the riboflavin was given daily in the form of a standard solution added to the breakfast fruit juice.

cola beverages, plain hard candy, black coffee and tea allowed ad libitum.

The subjects were college women 19 to 25 years old and weighing 47 to 74 kg. Five participated in the first year and 6 in each of the other years. All were judged by a preliminary physical examination to be in good health. The students lived in University residences or in private homes and made no changes in their usual activities during the experiment. Meals were prepared and served in the Nutrition Laboratory.

Analyses for calcium, phosphorus, riboflavin and nitrogen were made on 4-day composites of food, feces and urine. The food composites contained aliquots of tap water consumed as well as of all foods. Twenty-four-hour urine samples were preserved with chloroform and acetic acid; and feces, collected between carmine markers, were covered with 10% sulfuric acid until collections were complete. All samples were refrigerated until analyses were finished. Calcium and phosphorus were determined on the urines directly and on food and feces after dry ashing by slight modifications of the methods of McCrudden ('11–'12) and of Briggs ('22, '24). Riboflavin determinations were made by the fluorometric method of the Association of Vitamin Chemists ('51) and nitrogen analyses by the macro-Kjeldahl method using Hengar crystals as the catalyst.

RESULTS AND DISCUSSION

Calcium

The data obtained in the study of calcium utilization are reported in table 1. Mean urinary excretions during the restricted periods are identical and mean fecal excretions very similar, indicating that availability of the low level of calcium was not influenced by the diet whether it lacked only milk or was restricted in all three basic foods. For this reason the results will be considered as a whole rather than in terms of the particular foods omitted.

TABLE 1

Utilization of calcium and phosphorus on restricted diets, mineral- and vitamin-supplemented diets, and adequate diets of natural foods

DIET	CALCIUM			PHOSPHORUS		
	1950–'51	1951–'52	1952–'53	1950–'51	1951–'52	1952–'53
	gm/24 hr	*gm/24 hr*	*gm/24 hr*	*gm/24 hr*	*gm/24 hr*	*gm/24 hr*
Restricted						
Intake	0.322	0.358	0.264	0.885	0.868	0.785
Urine	0.148 ± .003 [1]	0.142 ± .011 [1]	0.140 ± .014 [1]	0.600 ± .021 [1]	0.517 ± .023 [1]	0.494 ± .029 [1]
Feces	0.252 ± .040	0.338 ± .030	0.239 ± .040	0.266 ± .034	0.335 ± .029	0.280 ± .052
Absorption	0.070 ± .040	0.020 ± .030	0.025 ± .040	0.619 ± .034	0.533 ± .029	0.505 ± .052
Retention	— 0.078 ± .031	— 0.130 ± .039	— 0.113 ± .034	0.019 ± .058	0.021 ± .043	0.010 ± .038
Supplemented						
Intake	1.446	0.980	1.386	1.699	1.349	1.739
Urine	0.226 ± .011	0.195 ± .012	0.170 ± .007	0.806 ± .025	0.674 ± .025	0.822 ± .030
Feces	1.153 ± .050	0.908 ± .034	1.207 ± .046	0.651 ± .043	0.667 ± .032	0.842 ± .059
Absorption	0.293 ± .050	0.072 ± .034	0.179 ± .046	1.048 ± .043	0.682 ± .032	0.897 ± .059
Retention	0.067 ± .040	— 0.123 ± .045	0.003 ± .039	0.242 ± .080	0.030 ± .049	0.075 ± .044
Natural foods						
Intake	1.459	1.024	1.294	1.618	1.285	1.449
Urine	0.195 ± .011	0.177 ± .012	0.200 ± .007	0.891 ± .025	0.764 ± .025	0.811 ± .030
Feces	0.951 ± .050	0.726 ± .034	1.041 ± .046	0.552 ± .043	0.514 ± .032	0.593 ± .059
Absorption	0.508 ± .050	0.298 ± .034	0.253 ± .046	1.066 ± .043	0.771 ± .032	0.856 ± .059
Retention	0.314 ± .040	0.122 ± .045	0.053 ± .039	0.175 ± .080	0.006 ± .049	0.046 ± .044

[1] Standard error of mean.

All subjects were in negative calcium balance throughout each of the restricted periods. The negative balance was to be expected since the low intake represented a marked decrease in the level of calcium habitually ingested by the young women. The male subjects studied by Hegsted, Moscoso and Collazos ('52) who were able to maintain equilibrium or even to achieve calcium storage on comparable levels of intake previously had lived on low-calcium diets for long periods of time.

The addition of $CaHPO_4$ to the restricted diet to give a total calcium intake of approximately one gram per day (1951–'52) failed to bring the subjects into equilibrium. All but one continued to lose calcium from body stores at approximately the same rate. Increase in the amount of the $CaHPO_4$ supplement to give a total daily calcium intake of 1.4 gm brought one group of subjects (1952–'53) into equilibrium on the average and the other (1950–'51) into slightly positive balance. One subject in each group was able to store 0.2 gm of calcium per day while another continued to lose calcium as rapidly as before. When similar amounts of calcium were supplied entirely from natural foods, storage of the mineral resulted. In each case availability of the calcium from food sources was greater ($P < 0.01$) than from $CaHPO_4$. The results indicate that young women can be maintained in calcium equilibrium on diets devoid of milk by supplementation of the restricted diet with $CaHPO_4$. However, the total calcium intake must be higher when most of the calcium comes from the mineral source.

Comparison of published data suggests that utilization of calcium from $CaHPO_4$ may be influenced by the age of the subjects or by the relative amounts of calcium from food and mineral sources. The work of Stearns and Jeans ('34–'35) and of Kempster et al. ('40) indicated that calcium from $CaHPO_4$ was as well utilized as milk calcium by preschool children. The latter authors reported approximately 20% utilization of both milk and $CaHPO_4$ supplements by young boys as compared to 32% utilization of milk calcium in young

men studied by Steggerda and Mitchell ('41, '46a). Utilization by our subjects of calcium from food supplements averaged 28% but from $CaHPO_4$ only 6%. Leichsenring et al. ('51) found that calcium added as a mixture of $CaHPO_4$ and $CaCO_3$ plus Na_2HPO_4 to low-calcium diets was poorly utilized by college women. However, in the diet of the preschool boys only 25% of the total calcium was supplied by $CaHPO_4$; in the studies of Leichsenring and those reported here 70 to 75% came from the mineral source.

Leichsenring and her associates found that addition of 1.2 gm of calcium to a basal diet supplying 0.3 gm of calcium and 0.8 gm of phosphorus produced greater calcium retention when the supplement was $CaCO_3$ than when a mixture of $CaHPO_4$ and $CaCO_3$ plus Na_2HPO_4 was used. Mean daily calcium retentions were 0.206 and 0.072 gm respectively. The authors interpreted their results as indicating that increased phosphorus intake adversely influenced calcium utilization and suggested that the amount of phosphorus normally present in the diet may be an important factor in the relatively poor calcium utilization in humans. Comparison of their data with the results of the present experiment suggest another interpretation. The Leichsenring carbonate-phosphate- and our 1950–'51 $CaHPO_4$-supplemented diet were very similar, and daily calcium retentions by the two groups of subjects were identical (0.072 and 0.067 gm). However, when essentially the same amounts of calcium and phosphorus were supplied entirely from natural-food sources, the retention of calcium by the Tennessee subjects was better (0.314 gm) than that of the Leichsenring group (0.206 gm) on the diet containing only 0.8 gm of phosphorus. The results seem to indicate that the source of calcium and phosphorus rather than the level of dietary phosphorus may influence calcium utilization in humans.

The difference in retention of calcium from the mineral and natural foods appears to be the result of differences in the absorption of calcium from the intestinal tract. Absorption as used here means simply the difference between intake and

excretion in the feces. While it is recognized that fecal calcium consists of not only unabsorbed dietary calcium but also considerable quantities secreted into the intestine (Steggerda and Mitchell, '46b; Stearns, '50; Nicolaysen et al., '53), and that the amount secreted may be influenced by the calcium status of the individual (Hansard and Plumlee, '54), an attempt to differentiate between dietary and endogenous calcium seems unprofitable in this case. It is evident that the amount of calcium absorbed by subjects on the mineral-supplemented diet (table 1) was less ($P < 0.01$) than from natural foods. Utilization was influenced only by the amount absorbed, regression coefficients of 0.91 ± 0.05 and 0.90 ± 0.06 being obtained for retention of absorbed calcium from the mineral-supplemented and natural-food diets respectively.

Phosphorus

On the restricted diets furnishing 800 to 900 mg of phosphorus average retention was approximately equilibrium (table 1). Two subjects were in negative balance throughout the restricted periods but 5 others achieved considerable storage. When the daily intake was almost doubled by addition of $CaHPO_4$ or milk and the other basic foods to the restricted diet a corresponding increase in fecal and urinary excretion of phosphorus resulted. The percentage of the dietary phosphorus in the feces remained essentially unchanged when the additional phosphorus was entirely from food sources, average excretions being 35 and 39% of the intake on the restricted and natural-foods diets respectively. Fecal excretion of phosphorus on the mineral-supplemented diets represented 46% of the intake. These results indicate that absorption of phosphorus, like that of calcium, was less efficient when much of the intake was from the mineral source. Urinary excretion was directly related to the amount of phosphorus absorbed at all levels of intake ($b = 0.52 \pm 0.03$). Utilization of the phosphorus absorbed was not influenced by the type of diet, retention being positive and the same for the mineral-sup-

plemented diets ($b = 0.64 \pm 0.07$) and the diets of natural foods ($b = 0.64 \pm 0.05$).

Although individual subjects differed considerably in the amount of phosphorus required to produce equilibrium the results seem to indicate that approximately 0.8 gm of phosphorus represents a minimal adequate intake for normal young women. The subjects in 1952–'53, who were taller and heavier than those in 1950–'51, failed on the average to store as much calcium or phosphorus from the supplemented diets. This will be discussed elsewhere.[3] Leichsenring et al. ('51) concluded that a daily phosphorus intake of 800 mg was adequate for young college women since an increase in the phosphorus intake by supplementation with $CaHPO_4$ did not result in increased retention in their subjects. McKay et al. ('42) observed that at phosphorus intakes below 1.1 gm mean retentions by college women were greater on controlled than on self-selected diets; Ohlson and her associates ('52) found that an intake of 1.25 gm per day was required for women in the 30 to 39 year age group on self-selected diets and that the requirement appeared to increase somewhat with age. The range of intakes described as adequate by different authors may be explained by the variations in phosphorus retention among individuals and in the same individual at different times resulting in part from the nature of the diet and the mineral status of the subjects.

Riboflavin

In spite of the general assumption that intestinal synthesis of riboflavin prevents relation of fecal excretion to intake, determination of fecal as well as urinary excretions by subjects on the three types of diet seemed worthwhile. The data[4] are presented in table 2 and in figures 1 and 2, the values being

[3] Williams, McDonald, Morrell, Schofield and MacLeod, in preparation.

[4] In 1951–'52, without the knowledge of the authors, one subject (M.N.J.) was receiving a vitamin capsule containing riboflavin on advice of her physician. Data for this subject are omitted from mean values in table 2 but are included in figures 1 and 2.

calculated per kilogram of body weight because of the possible influence of body size upon riboflavin requirements.

On both the mineral-vitamin-supplemented and natural-foods diets fecal excretion of the vitamin increased with intake, regression being significant in each case. Since the regression coefficients were not different ($b = 0.42 \pm 0.10$ and 0.23 ± 0.08) it seems probable that if intestinal synthesis of riboflavin took place at daily intakes of approximately 2 mg and above the synthesis was not influenced by difference in the type of diet. The fact that, at the low levels of intake furnished by the restricted diets, fecal excretion was only slightly lower than on the supplemented diets suggests possible intestinal synthesis at these low levels of riboflavin intake. Hathaway and Lobb ('46) found total fecal excretions on a diet of natural foods approximately three times that on a synthetic diet. Other investigators (Davis et al., '46; Oldham et al., '47) concluded that fecal excretion of riboflavin was not influenced by the level of intake or by changes in the nature of the diet. Results of the present study seem to indicate that the excretion was influenced by the level of intake but not by the differences in the nature of the diets represented.

Urinary excretion of riboflavin appeared to be directly related to intake with no evidence of influence by the nature of the diets used. Excretion on the restricted diet was approximately 20% of the intake; as the intake was increased urinary excretion rose to 28% of 2 mg and 36% of 2.6 mg of riboflavin daily. On intakes of 3.0 to 3.5 mg average excretion represented 25% of the daily intake. One subject, M. N. J.,[4] excreted 41% of the daily intake of 3.88 mg. The male subjects studied by Horwitt et al. ('50) excreted 9% of intakes up to 1.1 mg, 30% of 1.6 to 2.55 mg and 50% of a daily intake of 3.55 mg of riboflavin. The authors concluded that the critical riboflavin requirement of the adult male lies between 1.1 and 1.6 mg. Similarly, other authors (Brewer et al., '46; Oldham et al., '50) concluded from studies on women that to describe increases in urinary output with increasing intake of the vitamin it was necessary to fit two regression lines to the

TABLE 2

Utilization of riboflavin and nitrogen on restricted diets, mineral- and vitamin-supplemented diets, and adequate diets of natural foods

DIET	RIBOFLAVIN			NITROGEN		
	1950–'51	1951–'52	1952–'53	1950–'51	1951–'52	1952–'53
	mg/kg/24 hr	*mg/kg/24 hr*	*mg/kg/24 hr*	*gm/24 hr*	*gm/24 hr*	*gm/24 hr*
Restricted						
Intake	1.47 mg	1.21 mg	0.99 mg	10.63 gm	9.89 gm	9.90 gm
Av. intake/kg	0.026	0.021	0.016	...	...	...
Urine	0.005 ± .001 [1]	0.004 ± .001 [1]	0.004 ± .001 [1]	8.23 ± .27 [1]	7.91 ± .14 [1]	7.59 ± .14 [1]
Feces	0.019 ± .001	0.015 ± .000	0.018 ± .002	1.13 ± .05	0.98 ± .04	1.29 ± .05
Retention	0.002 ± .003	0.002 ± .003	— 0.005 ± .003	1.27 ± .26	1.00 ± .29	0.98 ± .24
Supplemented						
Intake	2.64 mg	1.88 mg	2.63 mg	11.20 gm	10.57 gm	10.73 gm
Av. intake/kg	0.046	0.033	0.044	...	...	...
Urine	0.016 ± .001	0.009 ± .001	0.017 ± .001	8.06 ± .30	7.91 ± .15	7.85 ± .16
Feces	0.024 ± .002	0.013 ± .001	0.022 ± .002	1.21 ± .16	0.93 ± .05	1.44 ± .06
Retention	0.005 ± .002	0.011 ± .003	0.005 ± .003	1.97 ± .31	1.72 ± .33	1.43 ± .27
Natural foods						
Intake	3.50 mg	1.98 mg	3.06 mg	11.12 gm	9.98 gm	10.51 gm
Av. intake/kg	0.061	0.035	0.051	...	...	...
Urine	0.016 ± .001	0.010 ± .001	0.013 ± .001	8.86 ± .30	8.40 ± .15	8.10 ± .16
Feces	0.019 ± .002	0.014 ± .001	0.023 ± .002	1.11 ± .16	0.88 ± .05	1.18 ± .06
Retention	0.026 ± .002	0.011 ± .003	0.014 ± .003	1.15 ± .31	0.71 ± .33	1.07 ± .27

[1] Standard error of mean.

data, one for excretion at intakes below 1.5 mg per day and the other to show the excretions obtained at higher levels of intake. This was not the case in the present study. The regression coefficient calculated for excretion at intakes below 0.035 mg ($b = 0.25 \pm 0.04$) was not significantly different from that obtained at the higher levels of intake ($b = 0.30 \pm 0.05$). Although urinary excretion of riboflavin by individual subjects (fig. 1) showed considerable variation, the average output at all levels of intake studied seems equally

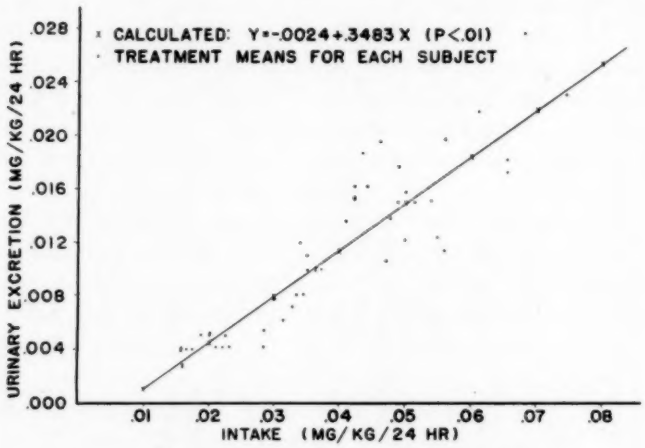

Fig. 1 Urinary excretion of riboflavin.

well described by the regression coefficient ($b = 0.35 \pm 0.02$). Urinary excretion of riboflavin, therefore, failed to indicate that a critical level of intake for these young women was reached up to the maximum of 3.5 mg per 24 hours.

Since both urinary and fecal excretions of riboflavin seemed directly related to intake, retentions were calculated. The average values for each group of subjects are presented in table 2 and for individual subjects in figure 2. It will be seen that retention increased significantly with intake without any indication that maximum retention had been reached. Such data suggest that these young women were able to utilize

dietary riboflavin at levels well above the recommended 1.4 to 1.9 mg per day. Data obtained by Sebrell and his associates ('41) with adult women were interpreted to indicate that 0.035 mg of riboflavin per kilogram of body weight was somewhat below the adult requirement and that 0.06 mg per kilogram is slightly more than the amount required. On this basis they suggested that 3 mg per day would approximate

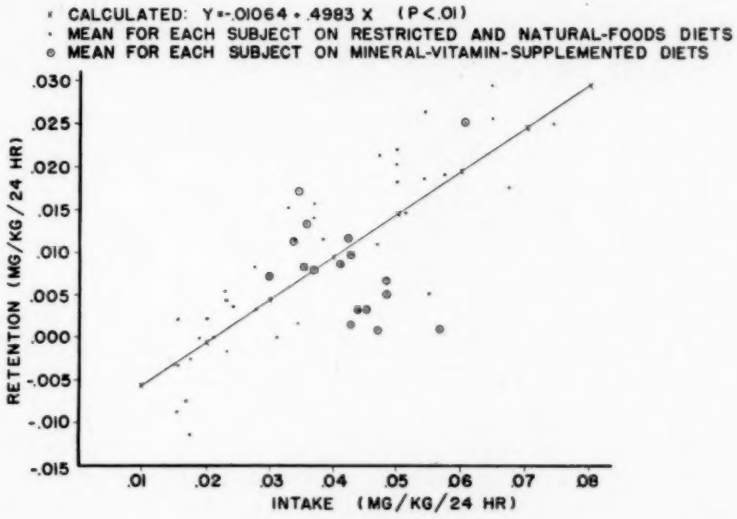

Fig. 2 Riboflavin retention.

the adult requirement. In the present study, the average retention of an intake of 0.06 mg per kilogram appeared to be equally as efficient as that observed at lower levels.

Greatest variability in retention by different subjects was observed on the mineral- and vitamin-supplemented diets (fig. 2). In only one of the years was the increase in riboflavin retention from the synthetic supplement significant, although all subjects appeared able to utilize some of the vitamin. Retention of riboflavin supplied by natural-food supplements

appeared more efficient and was greater ($P < 0.01$) than that given as the synthetic vitamin.

Nitrogen

All diets were planned to supply at least 60 gm of protein daily to insure a thoroughly adequate intake. The restricted diets contained considerably larger amounts of meat than did the diets which supplied three to 4 cups of milk daily. When wheat was omitted from the restricted diet in 1952–'53, a variety of other cereals was substituted. Consequently the proportions of animal and vegetable protein were essentially the same throughout the experiment. Variations in protein intake from period to period were kept as small as was possible when the diet was designed to include a good variety of foods. The maximum difference in nitrogen intake for any two balance periods in the three years of the experiment was 2.5 gm per 24 hours.

Although these differences in nitrogen intake were small and varied from period to period, increase in intake produced a significant increase in retention ($b = 0.67 \pm 0.22$). Extrapolation of the regression line indicates that a daily intake of 8.62 gm of nitrogen or 54 gm of protein was required to maintain these subjects in equilibrium. Except for an occasional slightly negative balance exhibited by an individual subject, nitrogen balances were positive throughout the experiment. Mean values for excretion and retention of nitrogen by all subjects in each treatment are recorded in table 2. The data are similar to those generally reported by other authors.

SUMMARY

On diets devoid of milk, eggs and wheat and furnishing approximately 300 mg of calcium, 800 mg of phosphorus and 1.0 to 1.5 mg of riboflavin per day, all subjects were in definitely negative calcium balance while mean retentions of phosphorus and riboflavin approached equilibrium.

Supplementation of the restricted diets with dicalcium phosphate and riboflavin or with milk, eggs and wheat gave daily intakes of 1.0 to 1.4 gm of calcium, 1.3 to 1.7 gm of phosphorus and 1.9 to 3.5 mg of riboflavin. Utilization of all three nutrients was more efficient when the supplement was supplied by natural foods than by the mineral and synthetic vitamin. The decreased availability of calcium and phosphorus from the mineral source resulted from smaller absorption of calcium and phosphorus from the intestinal tract. The restricted diets became adequate to maintain all subjects in positive balance with respect to calcium and phosphorus when the $CaHPO_4$ supplement raised the daily calcium intake to approximately 1.4 gm.

Riboflavin excretions appeared to be directly related to intake. Retentions increased significantly with intake without any indication that maximum retention had been reached. The data were interpreted to mean that these young women were able to utilize dietary riboflavin at levels well above the recommended 1.4 to 1.9 mg per day.

Mean nitrogen retentions were positive throughout all periods with daily intakes of 9.9 to 11.1 gm. Extrapolation of the regression line for retention on intake indicated that 8.6 gm of nitrogen or 54 gm of protein per day was required to maintain equilibrium in these subjects.

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BENEFICIAL EFFECTS OF ALFALFA, AUREOMYCIN AND CORNSTARCH ON THE GROWTH AND SURVIVAL OF HAMSTERS FED HIGHLY PURIFIED RATIONS[1]

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Data are available indicating that hamsters will grow and survive on highly purified rations containing casein as the dietary protein and either glucose or sucrose as the dietary carbohydrate (Cooperman, Waisman and Elvehjem, '43; Hamilton and Hogan, '44; Granados, '51). In attempts to duplicate these findings, however, a marked variation in the growth and survival of hamsters obtained from different breeders was observed when these animals were fed purified diets containing the above ingredients. Whereas hamsters obtained from one commercial breeder showed excellent growth and survival on such diets, animals obtained from two other suppliers failed to survive on identical rations although hamsters from all three sources grew equally well when fed a natural food ration. These findings and the effects of various dietary modifications are presented in the present communication.

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PROCEDURE AND RESULTS

Three basal rations which differed only in their carbohydrate component were employed in the present experiment. Diet A contained sucrose; diet B, glucose; and diet C, corn starch as the source of carbohydrate. The composition of these rations was as follows: carbohydrate, 64%; casein[2], 24%; salt mixture,[3] 5%; and corn oil, 7%. To each kilogram of the above diets were added the following crystalline vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2-methyl-naphthoquinone, 5 mg; and choline chloride, 2 gm. To each kilogram of diet were also added 5000 U.S.P. units of vitamin A, 500 U.S.P. units of vitamin D_2 and 100 mg of alpha-tocopherol acetate. The vitamins were added in place of an equal amount of carbohydrate.

Forty male and 40 female hamsters ranging from 30 to 40 gm in weight were obtained from each of three breeders, one of whom was located in New York,[4] the other two in California.[5,6] Hamsters from each source were divided into 4 equal groups consisting of 10 males and 10 females each. The animals were placed in metal cages with raised screen bottoms and were fed the following rations ad libitum: (1) diet A, (2) diet B, (3) diet C and (4) a natural food stock ration.[7] The experimental diets were made up weekly and stored under refrigeration when not in use. The animals were fed daily, and all food not consumed 24 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. Feeding was continued for 5 weeks or until death, whichever occurred first.

[2] Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Hubbell, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Tumblebrook Farm, Brant Lake, New York.

[5] West Side Hamstery, Tracy, California.

[6] Mission Animal Supply, San Gabriel, California.

[7] Ground Purina Laboratory Chow, Ralston Purina Co., St. Louis, Missouri.

A marked difference in response was observed between hamsters obtained from the three breeders.[8] All hamsters obtained from the New York supplier survived the experimental period of 5 weeks with an average weight increment of 40 to 48 gm for animals in the various dietary groups. In contrast the hamsters obtained from the two California suppliers failed to survive when fed diets A and B although their growth and survival were similar to that of the New York hamsters when fed diet C or the natural food ration. In general the California hamsters grew well on all 4 diets for the first 7 to 10 days of feeding. Thereafter growth was retarded or actual loss in body weight occurred in most of the animals fed the glucose or sucrose-containing rations (diets A and B) although no impairment in growth occurred on the cornstarch-containing or natural food ration. Approximately 80% of the hamsters obtained from the California suppliers and fed diets A or B died within the first 5 weeks of feeding after an average survival time of 16 to 24 days. Similar findings were obtained with both male and female animals. In contrast only three out of 40 California hamsters fed diet C died during the experimental period with no deaths occurring among an equal number of animals fed the natural food ration.

Experiments were subsequently conducted on the effects of the following modifications of diets A and B on the survival of hamsters obtained from the California suppliers: (1) incorporating roughage in the above diets at 2 and 5% levels in the form of agar, cellophane or cellulose, (2) substituting cottonseed oil, olive oil, wheat germ oil or lard for the corn oil in the ration, (3) substituting cod liver oil for the synthetic vitamins A and D_2 in the ration, (4) substituting dextrin for the glucose or sucrose in the ration, (5) doubling the amount of crystalline B vitamins, ascorbic acid, fat-soluble vitamins

[8] No data are available to account for the diverse findings obtained for hamsters from the different suppliers. Such factors as differences in the pre-test dietary regime, strain differences in nutritional requirements or differences in the intestinal flora may all have contributed to the diverse results.

or fat in the diet, (6) adding supplements of casein,[9] yeast [10] or desiccated whole liver powder [11] at a 10% level in the diet or (7) incorporating small amounts of the feces of the hamsters obtained from the New York supplier after such hamsters were maintained for at least two weeks on diets A or B. The various supplements were added in place of an equal amount of carbohydrate. Five male and 5 female hamsters from each of the California suppliers were fed each of the diets indicated above as well as the basal diets A and B. The hamsters were selected at 30 to 40 gm in weight and were fed the various rations ad libitum for 6 weeks or until death, whichever occurred first. No significant differences in growth, gross appearance or survival were observed between animals fed the above diets. From 60 to 100% of the hamsters of both sexes in all dietary groups died within the experimental period of 6 weeks. In contrast to the findings obtained with the above supplements, the incorporation of aureomycin HCl in diets A or B at a level of 100 mg/kg of diet or supplements of alfalfa meal at a 20% level in the diet resulted in a significant improvement in percentage survival. Eighty to 100% of the hamsters fed the latter supplements were still alive after 6 weeks of feeding.

The comparative effects of supplements of aureomycin HCl, alfalfa meal and various alfalfa fractions on the growth and survival of the hamsters obtained from the California suppliers are indicated in table 1. The findings indicate that aureomycin HCl at a level of 100 mg/kg of diet or alfalfa meal at a level of 20% of the ration when fed in conjunction with diets A and B were equally effective in promoting the growth and survival of hamsters under conditions of the present experiment. Similar findings were obtained with both male and female animals. No significant difference in activity was observed between the oven-dried meal and the sun-dried and vacuum-dried material. The active factor (or factors) in

[9] See footnote 2, page 580.

[10] Brewers' Type Yeast no. 200, Anheuser-Busch, Inc., St. Louis, Missouri.

[11] Desiccated Liver N.F., The Wilson Laboratories, Chicago, Illinois.

TABLE 1

Comparative effects of aureomycin HCl, alfalfa meal and alfalfa fractions on the growth and survival of hamsters fed highly purified rations (10 animals per group)[1,2]

DIETARY GROUP	SEX	BODY WEIGHT AFTER 6 WEEKS OF FEEDING	PER CENT SURVIVAL[3]	AVERAGE LENGTH OF SURVIVAL[4]
		gm		*days*
Experiment no. 1				
A	M	..	0	16
B	M	..	0	19
C	M	74	100	42
A + aureomycin HCl	M	90	100	42
B + aureomycin HCl	M	87	100	42
A + 20% oven-dried alfalfa[5]	M	88	70	37
B + 20% oven-dried alfalfa	M	88	100	42
Experiment no. 2				
A	F	76	30	24
B	F	..	0	17
C	F	83	80	38
A + aureomycin HCl	F	101	90	40
B + aureomycin HCl	F	102	100	42
A + 20% oven-dried alfalfa	F	86	100	42
B + 20% oven-dried alfalfa	F	95	80	40
Experiment no. 3				
B	M	..	0	26
B + aureomycin HCl	M	87	80	39
B + 20% oven-dried alfalfa	M	97	80	39
B + 20% sun-dried alfalfa	M	91	90	40
B + 12% alfalfa residue	M	99	100	42
B + 8% dried alfalfa juice	M	76	30	29
B + 2.5% alfalfa ash	M	91	20	26
Experiment no. 4				
B	F	66	10	25
B + aureomycin HCl	F	94	80	38
B + 20% oven-dried alfalfa	F	98	70	39
B + 20% sun-dried alfalfa	F	102	70	40
B + 12% alfalfa residue	F	99	80	39
B + 8% dried alfalfa juice	F	89	20	27
B + 2.5% alfalfa ash	F	72	20	31
Experiment no. 5				
A	F	..	0	16
B	F	..	0	18
C	F	101	90	40
A + aureomycin HCl	F	92	80	38
A + 20% oven-dried alfalfa	F	107	90	40
A + 20% sun-dried alfalfa	F	99	80	39
A + 20% vacuum-dried alfalfa	F	103	80	38
A + 12% alfalfa residue	F	106	90	40
A + 8% dried alfalfa juice	F	101	30	24
A + 2.5% alfalfa ash	F	81	20	20

[1] The hamsters employed in experiments 1 to 4 inclusive were obtained from the West Side Hamstery, Tracy, California. The hamsters employed in experiment 5 were obtained from the Mission Animal Supply, San Gabriel, California.

[2] The hamsters ranged from 30 to 40 gm in weight at the start of the experiment with an average weight of 34 to 36 gm for each of the dietary groups.

[3] Based on the number of animals alive after 6 weeks of feeding.

[4] Data were calculated on the basis of a 42-day survival time for animals alive at the termination of the experiment.

[5] The alfalfa and alfalfa fractions replaced equal amounts of carbohydrate.

alfalfa was retained in the alfalfa residue fraction (the water washed pulp remaining after the extraction of the juice). Dried alfalfa juice had little if any activity. Alfalfa ash when fed at a level corresponding to the amount present in 20% alfalfa meal was similarly ineffective.

DISCUSSION

Data are available indicating that alfalfa contains one or more factors, apparently distinct from any of the known nutrients, essential for optimal growth and physiological performance under various experimental conditions. The earlier literature in this field has been reviewed by Kohler ('53). Additional data indicating the presence of unidentified nutrients in alfalfa have been presented by Hansen et al. ('53), Fisher et al. ('54), Ershoff ('54), Ershoff et al. ('56) and others. The present findings indicate that alfalfa contains a factor or factors apparently distinct from any of the known nutrients which promoted the growth and survival of immature hamsters fed highly purified rations containing casein as the protein and either glucose or sucrose as the carbohydrate. The protective factor (or factors) in alfalfa was retained in the alfalfa residue fraction (the water washed pulp remaining after the extraction of the juice). Supplements of all the known nutrients were without significant effect. No data are available to indicate whether the protective effects of alfalfa in the present experiment were due to its content of one or more of the unidentified factors indicated above. Inasmuch as supplements of aureomycin HCl at a level of 100 mg/kg of ration or the substitution of cornstarch for the glucose or sucrose in the test rations were just as effective as supplements of alfalfa meal in promoting the growth and survival of hamsters under conditions of the present experiment, it is possible that cornstarch may also contain the protective factor or may promote (as may aureomycin) the synthesis of a similar or identical factor by the intestinal flora.

SUMMARY

A marked variation was observed in the response of hamsters obtained from different breeders in respect to growth and survival when fed highly purified rations containing casein as the protein and either glucose or sucrose as the carbohydrate. Whereas hamsters obtained from one breeder showed excellent growth and survival on highly purified rations containing the above ingredients, animals obtained from two other suppliers failed to survive on identical rations although hamsters from all three sources grew equally well when fed a natural food ration. The poor growth and survival of hamsters on the above diets could be ameliorated by the substitution of cornstarch for the glucose or sucrose in the ration, the addition of aureomycin HCl at a level of 100 mg/kg of ration or the incorporation of alfalfa meal at a 20% level in the diet. The protective factor (or factors) in alfalfa was retained in the alfalfa residue fraction (the water washed pulp remaining after the extraction of the juice). Supplements of all the known nutrients were without significant effect.

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A MICROBIOLOGICAL METHOD FOR THE NUTRITIONAL EVALUATION OF PROTEINS [1]

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Determinations of nutritional values of proteins have in the past depended to a great extent upon biological assays. The criteria for evaluation have included such responses as growth, maintenance, tissue regeneration, reproduction and lactation, in a variety of laboratory animals. Since the assays are relatively slow, costly and subject to individual differences, interest is developing in microbiological methods. In this connection Horn, Blum and Womack ('54) used growth of *Leuconostoc mesenteroides* to measure the change in value of a feed resulting from processing. The growth response of *Tetrahymena geleii*, a protozoan, was used by Anderson and Williams ('51) for evaluation of protein hydrolysates. This method was later applied to some unhydrolyzed proteins by Pilcher and Williams ('54). Growth responses were measured by photometric determination of the red triphenylformazan formed by the enzymatic reduction of colorless 2, 3, 5-triphenyltetrazolium chloride. A method by Halevy and Grossowicz ('53) involved digestion of proteins with pancreatin and subsequent testing of the growth-promoting activity of the hydrolysate for *Streptococcus fecalis*. Photometric measments of growth turbidity were used to estimate growth. A more recent method for determining protein digestibility

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(Mertz, Rennert and Cole, '55), combined the proteolytic action of pepsin with that of the bacterium *Pseudomonas aeruginosa.* Digestibility was estimated from photometric measurement of the color developed after treating the hydrolysate with a ninhydrin reagent.

While the use of a microbiological method for determining protein quality is at best a controversial matter, it is hoped that the procedure reported herein may contribute, in some degree, to our understanding of this complex situation. The method uses several enzymes normally employed in animal digestion and therefore is considered to measure more closely the availability of protein to animals as well as the value of the available portion. The assay is by measurement of the growth of *Streptococcus fecalis* in a medium having amino acids supplied by protein hydrolysates. An added advantage of the method is that growth measurement is by pH determination, thus obviating the extra steps often required to overcome the effects of contaminating turbidities, or color changes, when growth is measured turbidimetrically.

EXPERIMENTAL

The test organism was *Streptococcus fecalis,* obtained from the American Type Culture Collection under number 9790. Of the amino acids indispensable to man it requires lysine, tryptophan, threonine, leucine, and isoleucine. Of the partially indispensable ones it requires arginine and glycine and, for full growth, cystine and tyrosine. It also needs aspartic and glutamic acids, alanine and serine. Although *Leuconostoc mesenteroides* P. 60 has amino acid requirements even more closely related to those of the human, it was eliminated during preliminary trials because of lower sensitivity. Further improvement of this method would undoubtedly be effected by use of a microorganism, if one could be found, which has requirements more nearly like those of *L. mesenteroides* and yet which would perform as satisfactorily in the procedure as *S. fecalis.*

Purified proteins were subjected to enzymatic hydrolysis in a sequence paralleling human digestion. Five-gram samples were incubated for 24 hours at 37°C. in 150 ml of 0.5% pepsin solution adjusted to an initial pH of 1.8 with HCl. A layer of toluene was used during all digestions. On completion of the peptic digestion the solutions were adjusted to pH 8.4 and buffered with boric acid and potassium chloride. After the addition of 300 mg of 3 × U. S. P. pancreatin and 100 mg each of trypsin and a mixture of endopeptidases, commercially termed "erepsin"[2], the solutions were placed in the

TABLE 1

Composition of basal medium

COMPONENT	AMOUNT	COMPONENT	AMOUNT
	mg/100 ml		*mg/100 ml*
Sodium citrate	2500.00	Folic acid	0.001
Glucose	2000.00	Pyridoxine	0.012
Adenine	1.00	Biotin	0.00014
Guanine	1.00	K_2HPO_4	250.00
Uracil	1.00	KH_2PO_4	250.00
Thiamine	0.02	$MgSO_4 \cdot 7H_2O$	20.00
Riboflavin	0.02	NaCl	1.00
Nicotinic acid	0.06	$FeSO_4 \cdot 7H_2O$	1.00
Calcium pantothenate	0.04	$MnSO_4 \cdot 4H_2O$	1.00
Para aminobenzoic acid	0.001		

incubator for 72 hours. Frequently during the incubation the hydrolysates were well shaken to improve contact between enzymes and substrate. The hydrolysates were then adjusted to pH 7.0 and filtered to remove the bulk of undigested material. The controls, to which no protein was added, were also subjected to the same enzyme treatment.

The assay involved measuring acid production by *S. fecalis* after incubation in a basal medium containing 3% of hydrolysate as the sole source of amino acids. Five milliliters of basal medium (table 1), containing all required nutrients except amino acids, plus distilled water to make a final volume

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.

of 10 ml, completed the growth medium. After being autoclaved at 121°C. for 15 minutes all tubes were cooled, inoculated with one drop of a 24-hour broth culture of *S. fecalis*, incubated for 72 hours at 37°C., and the resultant acidities determined by pH measurement.

Paper chromatography was used to determine the qualitative distribution of amino acids in the hydrolysates. The ascending method (Williams and Kirby, '48), using phenol-water and collidine-lutidine-water as the resolving solutions, followed by ninhydrin spray, detected most of the amino acids shown to be present. It is to be noted that leucine and isoleucine are not separated by this method. Histidine was detected by the Pauli test, and tryptophan by the Ehrlich test, after resolution in butanol-acetic acid-water. Phenylalanine and methionine were separated from the other amino acids by three successive resolutions in butanol-acetic acid-water and were detected by reaction with isatin (Saifer and Oreskes, '54).

RESULTS AND DISCUSSION

Chromatography results are summarized in table 2 and indicate an extensive hydrolysis of each protein investigated. It is assumed that the enzymatic hydrolysis of each protein proceeded to an extent proportional to that protein's availability in animal nutrition. Measurement of acid production by *S. fecalis*, therefore, when grown in a medium the amino acid content of which is supplied solely by the hyrdolysate, is a convenient means of determining, on a comparative basis, the nutritive value of different proteins. Typical data are presented in table 3 accompanied, for purposes of comparison, by data which have been reported from other procedures.

It should be stressed that such results, dependent as they are upon pH measurements, are not necessarily absolute values, but do serve as a convenient means of comparing different proteins, and of rapidly classifying protein sources as good, poor or intermediate.

TABLE 2

Free amino acids in the hydrolysates [1]

AMINO ACIDS	CASEIN	ALBUMIN	GLUTEN	GELATIN	ZEIN
Aspartic acid	++	+++	++	++	+
Glutamic acid	+++	+++	++	++	+
Serine	+	+	+	+	+
Glycine	+	+	+	+++	+
Arginine	++	+	+	++	+
Lysine	+++	++	low	+++	low
Alanine	++	+++	++	++	+++
Threonine	++	++	+	+	+
Valine	+++	+++	++	++	++
Leucine (s)	++++	+++	+++	+++	++++
Tyrosine	++	+++	++	+	++
Methionine	+	++	low	trace	++
Tryptophan	++	+++	+	trace	trace
Histidine	?	?	?	low	+
Proline	+	?	?	?	+
Phenyl-alanine	++	++	++	+	+++
Asparagine	+	+	+	?	+

[1] + indicates present, ++ indicates present in greater amount, etc.

TABLE 3

Comparative evaluation of various proteins

PROTEIN	pH	6.9 − pH (a)	"BIOLOGICAL VALUE" $a \times \frac{100}{2.25}$ [1]	VALUES BY OTHER PROCEDURES (ALBUMIN = 100)		
				S. fecalis (turbidimetric) [2]	Growing rats [3]	Mature humans [4]
None	6.90					
Albumin	4.65	2.25	100	100	100	100
Casein	5.10	1.80	80	95	71	62
Gelatin	5.30	1.60	71	28		
Gluten	5.75	1.15	51	14	41	46
Zein	6.25	0.65	29	2		

[1] Factor to give albumin a "value" of 100.
[2] Halevy and Grossowicz ('53).
[3] Mitchell and Beadles ('50).
[4] Hawley et al. ('48).

It should be further pointed out that when crude protein sources are to be assayed, total nitrogen determinations should be carried out on the original materials, and the results calculated on the basis of equivalent amounts of total nitrogen.

The results in table 3 compare well with those reported by Mitchell and Beadles ('50) and by Hawley et al. ('48) from nitrogen balance experiments with growing rats and mature humans. They also compare well with those reported by Halevy and Grossowicz ('53), from a somewhat different microbiological procedure, except for "higher values" for the poorer quality proteins. It is felt that this may result from the more extensive enzyme hydrolysis by this method, and may more nearly represent the true availability, for animals, of the proteins.

SUMMARY

A relatively simple and rapid method for the comparative nutritional evaluation of proteins uses the enzymes normally employed in animal digestion and thus measures availability as well as value of the available portion. Assay is by measurement of acid production by *Streptococcus fecalis* is a medium having amino acids supplied by protein hydrolysates.

Chromatographic analysis of the hydrolysates serves to indicate the extent of the hydrolytic process, and also shows which amino acids are lacking, or are present in limited amounts, in the poor quality proteins.

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BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1957 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. The award is usually given to one person, but if in their judgment circumstances and justice so dictate, the Jury of Award may recommend that it be divided between two or more collaborators in a given research. The Jury may also recommend that the award be omitted in any given year if in its opinion the work submitted does not warrant the award. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award. Employees of the Borden Company are not eligible for this honor.

Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award

Chairman, Nominating Committee:
C. G. MACKENZIE
Department of Biochemistry
University of Colorado School of Medicine
4200 E. 9th Avenue, Denver 7, Colorado

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award.

Chairman, Nominating Committee:
R. V. Boucher
Agricultural and Biological Chemistry
Pennsylvania State University
University Park, Pennsylvania